though corneas of GDLD patients appear to be normal (fig. 1). This particular observation is always observed in both eyes of virtually all GDLD patients. However, it should be noted that the hyperfluorescence of the cornea is not limited to GDLD but is also observed in various conditions with corneal epithelial damage, including drug toxicity, dry eye, or atopic or vernal keratoconjunctivitis [16].

Genetics and Molecular Biology of Gelatinous Drop-Like Corneal Dystrophy

Identification of the Causative Gene for GDLD

When investigators searched for the responsible gene for GDLD, the TGFBI gene, a causative gene for various types of corneal epithelial-stromal dystrophies [17], was first examined; however, no pathological mutations were found in any of the patients with this disease [18]. Mutation analysis of the lactoferrin gene was also performed, but no pathological mutations were found in this gene [19]. In 1998, Tsujikawa et al. [20] performed a linkage analysis of 10 consanguineous Japanese families with a total of 13 affected members and found genotype-phenotype linkage with a maximum LOD score of 9.8 at the D1S2741 microsatellite marker on the short arm of chromosome 1. They also performed haplotype analysis and further defined the disease-associated locus within 2.6 cM (approximately corresponding to 2.6×10^6 bp) between the markers D1S2890 and D1S2801 [20]. Using 3 additional markers, they further narrowed the probable range to a 400-kb critical region between the markers D1S2648 and D1S2752 [21]. Subsequently, they isolated cosmid and BAC clones covering this 400-kb critical region and determined nearly its entire sequence by the shotgun cloning method. In that region, only 1 known gene was located. They performed a candidate gene approach for this gene and found 4 different mutations in the TACSTD2 gene (alternatively named as M1S1 or TROP2) in 26 patients of 20 Japanese GDLD families [21]. The 4 mutations include p.118Q→X, p.Gln211ArgfsX60, p.Gln207X, and p.Ser170X (originally designated as Q118X, 632delA, Q207X, and S170X, respectively). All of these are nonsense or frameshift mutations, thus producing truncated protein lacking a computationally predicted transmembrane domain located near the C-terminal region of this gene. Subsequently, Ren et al. [22] also performed linkage analyses of 8 unrelated GDLD families from different ethnic backgrounds (Indian, European, Tunisian) other than Japanese. They found that in 7 of those families the disease locus fell to a 16-cM region at the short arm of chromosome 1, which includes the TACSTD2 gene. They also performed mutation analysis of the TACSTD2 gene and identified 7 novel mutations in those families. The 1 remaining GDLD family was found to have no mutation in the TACSTD2 gene, suggesting the existence of other genes responsible for the occurrence of GDLD as reported in other studies [23–26].

From the same sets of patients as above, Tsujikawa et al. [20] also found linkage disequilibrium between the microsatellite marker D1S220 and the p.118Q→X

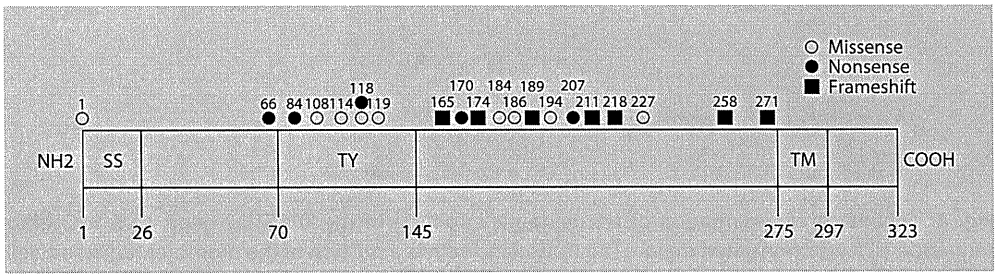

Fig. 3. Schematic representation of the distribution of TACSTD2 mutations and the domain structure of the TACSTD2 protein. SS = Signal sequence; TY = thyroglobulin-like domain; TM = transmembrane domain. All mutations are depicted with amino acid numbers and their effects on protein change such as missense (○), nonsense (●), and frameshift (■).

mutation, suggesting that GDLD patients bearing this nonsense mutation all descended from the same ancestor in whom the founder mutation occurred. Interestingly, the p.118Q→X mutation was also found in a Chinese GDLD patient [27]. Possibly, that patient's mutation descended from the putative founder mutation in common with all of the Japanese GDLD patients bearing the p.118Q→X mutation.

Mutation Analysis

After the determination of the TACSTD2 gene as the gene responsible for this disease, a large number of mutation studies have been made on GDLD patients in different countries with different ethnic backgrounds. At the time of writing, there have been 13 reports demonstrating 21 mutations comprised of 14 substitution, 4 deletion, and 2 insertion mutations, as well as 1 delins (a condition where insertion occurs in a deleted region) mutation, causing 9 missense, 6 nonsense, and 6 frameshift protein alterations in patients from 9 ethnic backgrounds including Japanese, Indian, Chinese, Iranian, Estonian, Turkish, Tunisian, Vietnamese, and European (table 1; fig. 3) [11, 12, 21–24, 27–33].

In spite of such allelic heterogeneity, most of the patients are homozygous and share only 1 mutation at both alleles, indicating that most GDLD patients were born to parents who are closely related. Actually, parents of GDLD patients demonstrate a frequency of consanguineous marriage of 43%, much higher than the frequency of 6.8% found in the general population [23]. Some of the parents who are not consanguineous may have the same TACSTD2 mutation in 1 of their alleles, especially when their birthplaces are close to each other. Consanguineous marriages may also be an important factor for the reason why GDLD frequently occurs in the Japanese population. In Japan, interfamily marriage (i.e. to a cousin) had historically been more common than in Western countries and is still currently permitted by law. This social tradition may have facilitated the occurrence of GDLD in this country. The fact that

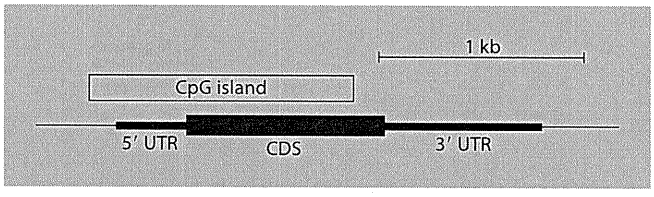

Fig. 4. Schematic representation of the genomic structure of the TACSTD2 gene. CDS = Coding sequence; UTR = untranslated region. The CpG island covers a portion of the promoter region as well as the 5′ UTR and more than 3/4 of the CDS of the TACSTD2 gene.

Japan is a relatively heavily populated country with an advanced national healthcare system may also be a factor as to why GDLD is more frequently seen in this country. Since the trend of consanguineous marriage is on the decrease in Japan, especially in recent years [34], it is expected that the incidence of GDLD in this country will gradually begin to mirror that decrease in the near future.

Although the number is small, the GDLD of some patients was reportedly caused by compound heterozygous mutations [21, 27, 30, 31], a rare condition where one mutation occurs in one allele and another mutation occurs in another allele. Such complicated situations suggest that most of the sporadic cases of GDLD may be born to nonconsanguineous normal parents, both bearing different TACSTD2 mutations from each other. With the recent decrease in the frequency of consanguineous marriage in Japan, the sporadic cases should eventually begin to outnumber the familial cases in this country.

Genomic Structure of the TACSTD2 Gene

TACSTD2 is a single-exon gene comprised of a central coding region flanked by 5′ and 3′ untranslated regions (fig. 4). There are 22 single nucleotide polymorphisms in this gene, which potentially affect the expression and function of this gene. The promoter region of this gene contains several consensus sequences which are potentially recognized by various transcription factors. Of note, TATA and GC box consensus sequences, which are respectively known as binding motifs for the transcription-initiating complex and ubiquitous transcription factor Sp1, are found at multiple sites upstream of the translation start site. The transcription start site for this gene has not been experimentally determined. However, expressed sequence tag analysis done for various purposes in research areas unrelated to ophthalmology imply the existence of 2 major transcription start sites located downstream of the 5′ end of a reference sequence (NM_002353, Refseq) of the TACSTD2 gene. Interestingly, more than half of the region of this gene, including its promoter, is significantly CpG rich (fig. 4). The so-called CpG island [35, 36] is often found in the upstream regions of many genes and is believed to be important for the regulation of gene expression. In general, when the cytosine residues of the CpG island are methylated, expression of the gene will

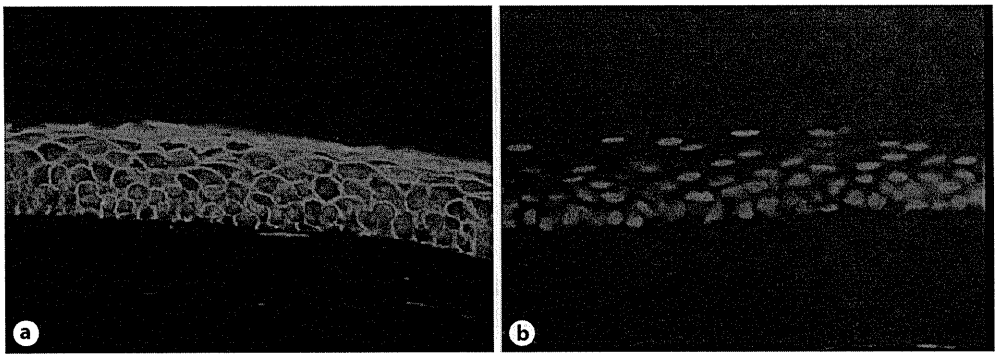

Fig. 5. A photograph representing immunolocalization of TACSTD2 protein in normal and GDLD corneal epithelia. Green signals = Immunoreaction to TACSTD2 protein; red signals = nuclei stained by propidium iodide. The TACSTD2 protein is localized at the cell-to-cell borders of almost all epithelial layers in normal cornea (**a**) but virtually completely absent in GDLD cornea (**b**).

be downregulated. Therefore, the existence of the CpG island in the TACSTD2 gene implies the potential epigenetic regulation for the expression of this gene. Resolution of these issues would appear to be fundamental in order to obtain a thorough understanding of the nature of the TACSTD2 gene; however, at the time of writing, virtually no experimental data have been presented that pertain to these issues.

Expression and Functions of the TACSTD2 Gene

In a study conducted in 1999, Northern blot analysis revealed that organs such as the cornea, placenta, lung, kidney, pancreas, and prostate express mRNA of the TACSTD2 gene [21]. TACSTD2 protein is thought to be a single-pass type I membrane protein consisting of 323 amino acids with a molecular weight of 35,709 Da. In that study, the authors also reported that forcedly expressed TACSTD2 protein was subcellularly localized to cytoplasm in COS-7 and HeLa cells. However, a subsequent study from another group has shown discrepant data that this protein was localized at the cell-to-cell border when forcedly expressed in CHO cells [31]. Human corneal tissue expresses this protein at the plasma membrane of all epithelial layers (our unpublished data, fig. 5). The protein functions of the TACSTD2 gene have yet to be fully elucidated, yet as one may imagine from the name 'tumor-associated calcium signal transducer 2', the gene is thought to be involved in cancer biology. In fact, there have been several reports regarding the relationship between this gene and cancers [37–39]. In these studies, the TACSTD2 gene has been reported to be highly expressed in many kinds of cancer cells and is thought to play an important role in the maintenance or facilitation of cancer cells. Interestingly, the epithelial cell adhesion molecule, the gene most homologous to the TACSTD2 gene, has also been reported to be highly expressed in many kinds of cancers [40–42] and has currently become an attractive and popular target for tumor therapy. In fact, there have been several clinical trials

utilizing monoclonal [43, 44] or bispecific antibodies [45, 46] and vaccination strategies [47] against this gene with varying degrees of success. Other than its relevance to cancer, the TACSTD2 gene was reported to be coupled with calcium signaling from the fact that the intercellular calcium concentration was dramatically changed when OvCa-432 cells were treated with anti-TACSTD2 antibody [48]. In addition, it has been demonstrated that in GDLD corneas, tight-junction-related proteins such as ZO-1, occludin, and claudin 1 are eliminated from the most apical side of the lateral junctions of the superficial cells [49], strongly suggesting that TACSTD2 protein may play an important role in the formation or maturation of the tight junction.

Computationally Predicted Attributes of TACSTD2 Protein

Several biological, chemical, and physical attributes can be computationally predicted for the TACSTD2 gene. SOSUI, a transmembrane domain prediction software [50], has shown that there are 2 potential transmembrane domains in this gene, and another transmembrane domain prediction software, one that employs the hidden Markov model algorithm (TMHMM) [51], also demonstrated nearly the same results. SignalP, a software used for predicting a signal sequence that is characteristic of secreted or membrane proteins [52], predicted the existence of a signal peptide and cleavage site at the N-terminus of TACSTD2 protein (fig. 3). Since the predicted signal peptide almost matches one of the transmembrane domains, the TACSTD2 gene supposedly has only 1 transmembrane domain near its C-terminus (fig. 3). PROSITE, a protein motif database [53], predicts that TACSTD2 protein has a thyroglobulin type 1 domain profile at its 70- to 145-amino acid region (fig. 3). PSORT, a software used for predicting the subcellular localization of proteins [54], implies that this protein is localized to the plasma membrane with a statistical certainty of 0.46, to the endoplasmic reticulum with a statistical certainty of 0.38, and to lysosomes with a statistical certainty of 0.20. The homologous gene for TACSTD2 can be determined by use of a homology search software such as BLAST [55]. By use of such software, it was discovered that the best-aligned gene is epithelial cell adhesion molecule, with a 50% similarity to the TACSTD2 gene.

Pathogenicity of TACSTD2 Mutations

Nearly two thirds of the mutations of the TACSTD2 gene that have so far been reported in GDLD patients are nonsense or frameshift mutations (deletion, insertion, and delins) which potentially lead to truncation of the TACSTD2 protein. It can easily be predicted that such alterations may lead to a change in the subcellular localization of this protein because all of these truncated TACSTD2 proteins lack the putative C-terminal hydrophobic transmembrane domain, and hence lose the capacity to be localized at the plasma membrane.

However, there have been some reports showing the existence in patients of GDLD caused by missense mutations of the TACSTD2 gene. Of such mutations, p.1M→R [22] is thought to be apparently pathological because this mutation will alter the start

codon methionine to arginine, and hence disrupt the translation initiation site where the ribosome apparatus will recognize and start translation. Therefore, in this case, the ribosome machinery will pass over the mutated translation initiation site, and hence its downstream ATG triplet may be recognized and work as an alternative translation initiation site. However, translation initiation generally requires not only the ATG triplet sequence but also a consensus sequence around the ATG triplet. The so-called 'Kozak' sequence is known to be the sequence, and this special sequence supposedly has the power to make the decision regarding translation strength [56, 57]. The Kozak sequence of the authentic translation initiation site of the TACSTD2 gene (–6 CCCACCAUGG +4) is nearly identical to the typical Kozak consensus sequence (–6 GCCA/GCCAUGG +4), suggesting that the translation strength of this gene is very high. As no potential Kozak sequences are found either upstream or downstream of the authentic Kozak sequence of the TACSTD2 gene, it is highly theorized that virtually no protein will be produced from the TACSTD2 gene bearing the p.1M→R mutation, although mRNA may be transcribed from this mutated TACSTD2 gene as high as the wild-type TACSTD2 gene.

Strictly speaking, it is still unclear as to whether other missense mutations are truly pathological or not. However, acceptable theoretical explanations have been given for some of those mutations. Frequently given explanations are that (1) the mutation causes amino acid transition with change in chemical properties such as from polar amino acid to nonpolar amino acid, or that (2) the mutation is found in affected family members but not found in unaffected family members in an affected pedigree (phenotype-genotype cosegregation), or that (3) the mutation is located on the putatively functional domains, thereby disrupting the protein function, or that (4) the mutation is found in GDLD patients but not found in normal volunteers, or that (5) the mutation is located at regions that are highly conserved among orthologous genes in animals and/or among paralogous genes in humans. A change in some specific amino acid is sometimes thought to be pathological. For example, mutations causing amino acid transition from cysteine to another amino acid [31] or a certain amino acid to cysteine [28] are expected to produce considerable impacts on the function of this gene, possibly through the disruption of the disulfide bond between 2 cysteine residues. However, since most single nucleotide polymorphisms in most genes are thought to be potentially nonpathological even if they carry changes in amino acid, such explanations are kinds of desk theories and they should be validated in the future through experiments.

Pathophysiology of the Gelatinous Drop-Like Corneal Dystrophy Cornea

Histology

Histologically, the GDLD cornea is characterized by subepithelial amyloid depositions, which can be recognized as an amorphous substance stained in red by

hematoxylin-eosin staining. The amyloid depositions are also specifically stained in red by Congo-red staining (fig. 6a), and those Congo-red-stained amyloid depositions demonstrate yellow-green birefringence when observed by a polarized microscope (fig. 6b). The corneal epithelium over the subepithelial amyloid depositions becomes thinner with a reduced number of epithelial layers and an absence of the Bowman's membrane. However, the corneal epithelium over the regions without amyloid depositions appears to be normal with normal thickness and the normal number of epithelial layers, as well as an intact Bowman's membrane [58]. Scanning electron microscopy also demonstrated that a larger number of desquamating cells were observed in the superficial cells of the GDLD cornea than in the normal cornea (fig. 6c) [7]. The scanning electron microscopy analysis also demonstrated that some of the apical intercellular junctions are loosened in the GDLD cornea (fig. 6d), while the normal cornea did not show such a prominent change [7]. Moreover, examination by electron microscopy indicated that horseradish peroxidase, a molecular tracer with a molecular weight of 40 kDa, permeated through the corneal epithelial barrier of a GDLD patient, while normal corneal epithelium did not allow this tracer to permeate into this tissue (fig. 6e) [59]. These observations, along with the clinically observed hyperfluorescence of the cornea in GDLD, are the main reason for our current hypothesis that epithelial barrier function may be severely damaged in the GDLD cornea.

Causative Protein for the Amyloid Depositions of the GDLD Cornea

In a study by Klintworth et al. [60], it was reported that a 78-kDa protein band exists in samples taken from GDLD corneas, whereas control samples did not show this protein band. In that study, amino acid sequencing analysis disclosed that the protein band was derived from lactoferrin. By Western blot analysis, the authors confirmed that the 78-kDa protein band is actually lactoferrin. Using an immunohistochemistry technique, they also found that the amyloid depositions in the GDLD cornea were really reacted with an antibody against lactoferrin. From these observations, they initially supposed that mutation of the lactoferrin gene may be responsible for the occurrence of GDLD; however, a subsequent study by that group showed that no mutations were found in the lactoferrin gene of GDLD patients [19].

Since an earlier study has demonstrated that apolipoproteins A and J exist in amyloid depositions [61], it is possible that not a single protein but, in fact, multiple proteins may participate in the formation of amyloid depositions of GDLD.

Pathogenesis of Gelatinous Drop-Like Corneal Dystrophy

Without question, the homozygous mutation of the TACSTD2 gene, which results in the loss of function of this gene, is definitely a primary reason for the occurrence

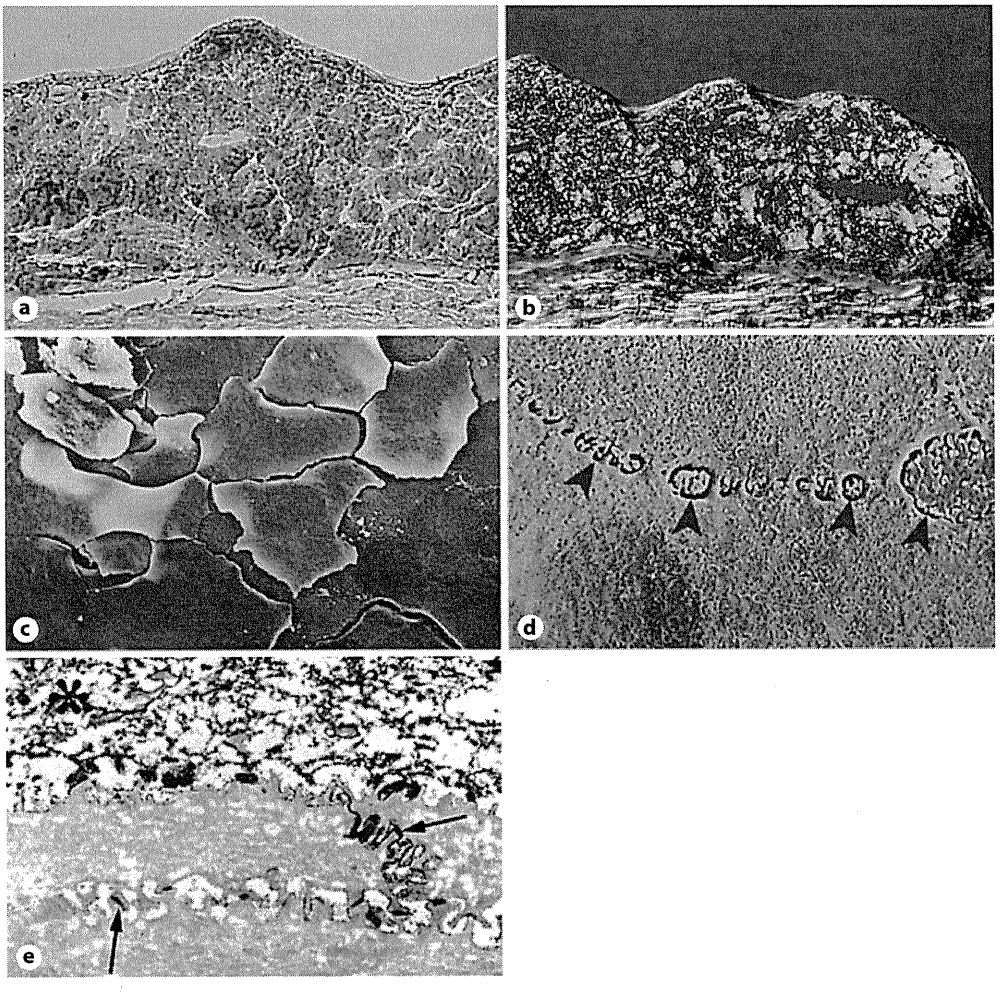

Fig. 6. An array of photographs showing histological examinations of GDLD cornea by hematoxylin-eosin staining (**a**), Congo red staining (**b**), Congo red staining by a polarized microscope (**c**), transmission electron microscopy (**d**), and scanning electron microscopy (**e**).

of GDLD. The decreased epithelial barrier function via the reduced expression of the epithelial tight-junction-related proteins seems to be the subsequent pathological event. The pathological event that next follows appears to be permeation of tear fluid through the loosened epithelial barrier function. Finally, the permeated tear lactoferrin may form amyloid depositions. This is our current concept for the pathogenesis of GDLD. However, there are still many aspects of this disease that have yet to be elucidated, issues such as why the loss of function of the TACSTD2 gene causes the reduced expression of the tight-junction-related proteins. In addition, it has yet to be explained why the lactoferrin protein, which is normally

water-soluble in the tear fluid, transforms into water-insoluble amyloid depositions when the protein permeates into corneal tissue. These 2 questions should be at the forefront of any future investigation aiming to formulate a complete understanding of this disease.

Treatment Strategies for Gelatinous Drop-Like Corneal Dystrophy

Surgical Strategies

Various types of treatments have been reported for GDLD. Currently, penetrating keratoplasty, deep lamellar keratoplasty, lamellar keratoplasty, and superficial keratectomy are the standard surgical procedures for this disease. However, almost all patients will develop recurrence within a few years after such interventions and repeated keratoplasties are frequently required for GDLD patients [62]. Therefore, less invasive procedures should be considered, especially for the patient's first surgical treatment. Complications associated with these treatments include glaucoma, infection, and rejection of the corneal graft. However, and although the reason for this phenomenon is currently unknown, there seems to be a tendency that steroid-induced glaucoma frequently occurs, and is much more severe, in GDLD. It is speculated that amyloid depositions may also exist in the trabecular meshwork of GDLD patients, and hence give those patients an inclination towards increased intraocular pressure. Since the loss of function of the TACSTD2 gene in the corneal epithelium is currently believed to be a primary reason for the pathogenesis of this disease, allogeneic transplantation of normal limbal tissue is sometimes considered [63]. This procedure completely replaces the patient's limbal epithelial cells with the donated ones. Thereafter, the transplanted corneal epithelial cells, which potentially have normal TACSTD2 gene function, will be regenerated from the transplanted limbal tissue, totally cover the patient's cornea, and result in epithelial barrier function that is nearly normal. Since the epithelial cells are more inclined towards allogeneic rejection than are corneal endothelial cells [64–66], high-dose immunosuppressive agents are frequently administrated to promote longevity of the transplanted limbal epithelial cells.

Contact Lens Wear

One promising alternative treatment for GDLD is soft contact lens wear. Although the underlying mechanism of how/why this treatment provides a beneficial effect on GDLD is currently unknown, we get the impression that the wearing of a soft contact lens actually retards the progression of this disease. The wearing of a soft contact lens may possibly decrease the local turnover of tear fluid around the corneal surface, thereby decreasing the permeation of tear fluid into corneal tissue. Alternatively, wearing of the lens may possibly enhance the physical integrity of the corneal epithelium, probably by protecting the epithelium from the shear stress that occurs at the time of blinking. Although it can be omitted in early GDLD cases, pretreatment by

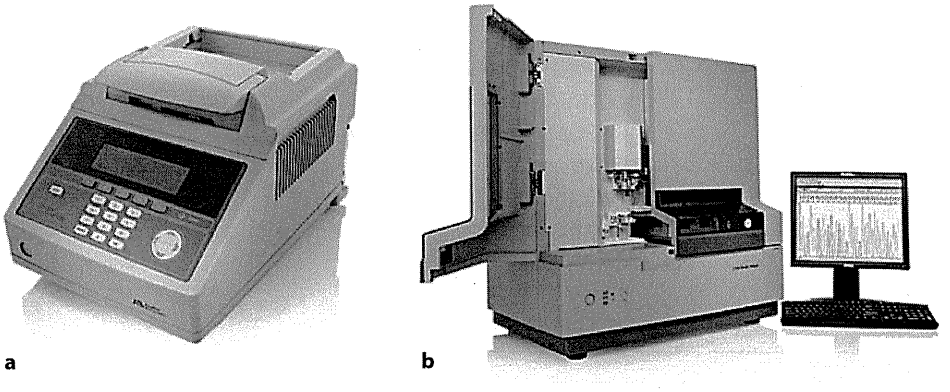

Fig. A1. An array of photographs representing equipments used in the molecular diagnosis of GDLD: thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems) (**a**) and automated sequencer (3130xl Genetic Analyzer, Applied Biosystems) (**b**).

excimer laser ablation using a smoothing technique [67] is preferable so that corneal protrusions are removed to alleviate foreign body sensation or pain during wearing of the soft contact lens.

Appendix: Laboratory System for the Molecular Diagnosis of Gelatinous Drop-Like Corneal Dystrophy

Since molecular diagnosis is frequently unavailable in most hospitals, researchers who intend to make an accurate diagnosis for patients with GDLD will often need to self-construct a system for molecular diagnosis. The minimum set of required equipment includes a thermal cycler (fig. A1a), an automated sequencer (fig. A1b), and a microcentrifuge. After setting up the required equipment, the first step is to extract genomic DNA from the patient's peripheral blood. This is most easily accomplished by use of a commercial column-based DNA extraction kit that is available from numerous companies. The second step is to amplify the extracted DNA using a primer pair which should surround the entire coding sequence of the TACSTD2 gene. Since the GC content of the coding sequence of this gene is relatively high (ca. 67.5%), it is beneficial to add 10% dimethyl sulfoxide into the PCR buffer, although it is highly dependent upon the experimental conditions. In our experimental condition, the 'touchdown' thermal condition [68] was found to be more preferable than the normal thermal condition. Tables A1 and A2 represent our current regimen for the PCR amplification of the TACSTD2 gene. The PCR product should be checked by electrophoresis on an agarose gel. After this confirmation, the PCR product should be purified by use of a commercially available purification kit to remove unreacted primers and dNTP because they will significantly reduce the efficiency of the subsequent sequencing reaction. There are many types of kits on the market specifically designed for this purpose. The most convenient purification kit may be a mixture of exonuclease I and shrimp alkaline phosphatase which cleaves the primer and dNTP, respectively. Then, the purified/treated PCR product can be used as a template for the sequencing reaction by the use of a commercial sequencing kit. The product of the sequencing reaction should be purified by a gel filtration (e.g. G50) or a simple ethanol precipitation. Then, the purified prod-

Table A1. The PCR parameters currently used for mutation analysis of the TACSTD2 gene

Forward primer	CCTGCAGACCATCCCAGAC
Reverse primer	CAGGAAGCGTGACTCACTTG
Enzyme	ExTaq
Buffer	1 × Vogelstein buffer
dNTP	1.25 mM
Mg^{2+}	3.9 mM
Additive	10% dimethyl sulfoxide
Thermal condition	touchdown

The Vogelstein buffer contains 67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 0.07% 2-mercaptoethanol and 0.067 mM EDTA.

Table A2. The thermal profile of touchdown PCR for the amplification of TACSTD2 gene

Step	Temperature/duration	Purpose	Repeats
1	94°C/3 min	denature	1
2	94°C/30 s, 70°C/1 min	amplification	3
3	94°C/30 s, 68°C/1 min	amplification	3
4	94°C/30 s, 66°C/1 min	amplification	3
5	94°C/30 s, 64°C/1 min	amplification	3
6	94°C/30 s, 62°C/1 min	amplification	3
7	94°C/30 s, 60°C/1 min	amplification	3
8	94°C/30 s, 58°C/30 s, 72°C/1 min	amplification	3
9	94°C/30 s, 55°C/30 s, 72°C/1 min	amplification	30
10	72°C/5 min	elongation	1

uct is ready for electrophoresis on an automated fluorescence sequencer. The obtained sequence data should then be analyzed by alignment software. Web-based free alignment software [e.g. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/)] is often sufficient for this purpose; however, well-designed, intelligent alignment software programs (e.g. Variant Reporter, Applied Biosystems) are available from some companies and are quite helpful for easy identification of gene mutations.

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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,

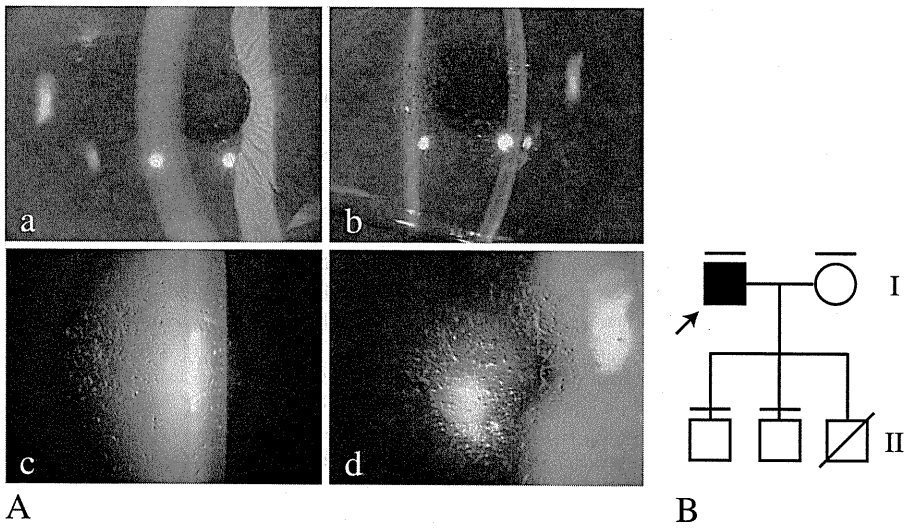

Figure 1 (A) Corneal phenotype of a lattice corneal dystrophy (LCD) patient with a p.Arg496Trp mutation. Photographs demonstrate dot- and lattice-like stromal depositions in the patient's corneas (A, C: right eye; B, D: left eye). (B) Schematic representation of the pedigree of the LCD patient.

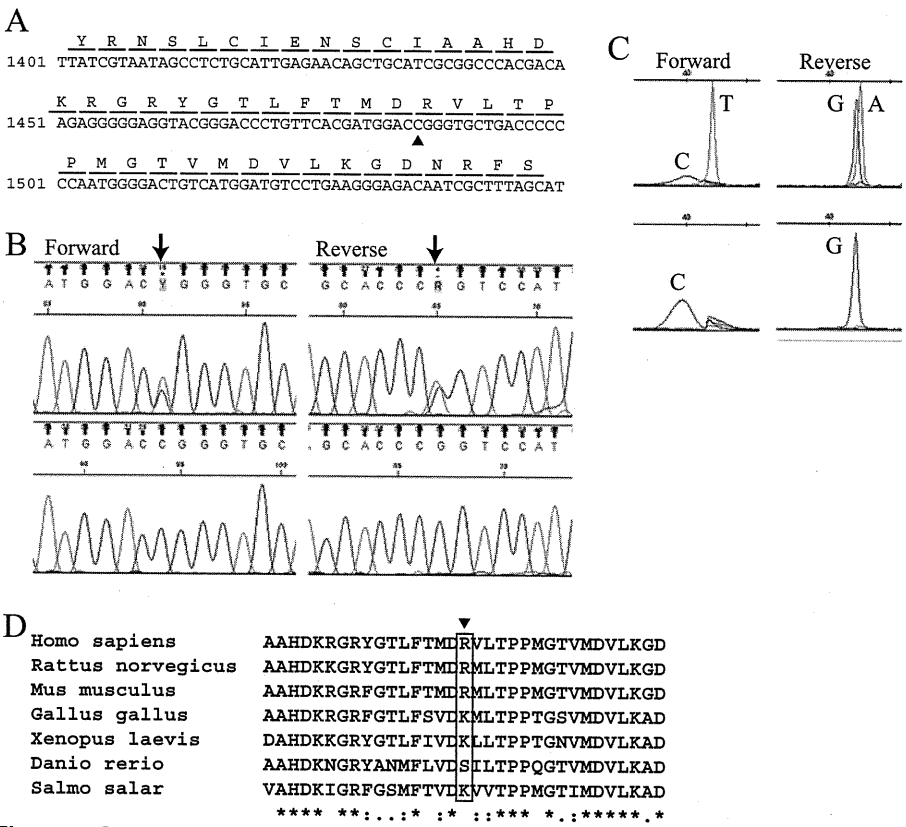

Figure 2 Results of sequencing and 1-base primer extension analyses for the LCD patient with a p.Arg496Trp mutation. (A) Nucleotide and amino acid sequence of the TGFBI gene. The arrowhead indicates the site of the c.1486C>T nucleotide change. (B) Results of sequencing analysis for the exon 11 of the 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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Two novel mutations of TACSTD2 found in three Japanese gelatinous drop-like corneal dystrophy families with their aberrant subcellular localization

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Purpose: To report two novel mutation of the tumor-associated calcium signal transducer 2 (*TACSTD2*) gene in 3 Japanese patients with gelatinous drop-like corneal dystrophy (GDLD).

Methods: Genomic DNAs were extracted from the peripheral blood of 3 Japanese families. The coding region of *TACSTD2* was amplified by polymerase chain reaction (PCR) and subjected to direct sequencing analysis. Plasmid vectors harboring normal and mutated *TACSTD2* were transfected to the immortalized human corneal epithelial cells to identify the subcellular localization of the normal and mutated *TACSTD2* gene products.

Results: Sequencing analysis of *TACSTD2* revealed two novel homozygous mutations (c. 840_841insTCATCATCGCCGGCCTCATC and c.675C>A which may result in frameshift (p.Ile281SerfsX23) and nonsense (p.Tyr225X) mutations, respectively) in the 3 GDLD patients. Protein expression analysis showed that the mutated gene product was distributed diffusely in the cytoplasm, whereas the normal gene product accumulated at the cell-to-cell borders.

Conclusions: This study reports two novel mutations in 3 GDLD families and expands the spectrum of mutations in *TACSTD2* that may cause pathological corneal amyloidosis.

Gelatinous drop-like corneal dystrophy (GDLD; OMIM 204870) was first described by Nakaizumi [1] as an uncommon, autosomal recessive disease, characterized by bilateral corneal amyloidosis. To date, this disease is still quite rare in many countries, however, it is relatively common in Japan with a prevalence rate of 1 in 31,546 individuals as estimated from the frequency of parental consanguinity [2,3]. In the first decade of the lives of GDLD patients, grayish, subepithelial nodular amyloid depositions appear and result in severe photophobia, lacrimation, and an ocular foreign body sensation [4,5]. As the patients age, the amyloid depositions typically enlarge, increase in number, coalesce, and exhibit a mulberry-like appearance, thus leading to severe bilateral vision loss usually beginning within the third decade of the patients' lives.

Tsujikawa et al. [6] revealed through the use of a linkage analysis and consecutive candidate gene approach that the specific gene responsible for this disease is tumor-associated calcium signal transducer 2 (*TACSTD2*). To date, fifteen reports have demonstrated twenty-three different GDLD-causing alterations in *TACSTD2* comprised of nine missense-, five nonsense-, and nine frameshift-causing (deletion and insertion) mutations from nine different geographical regions including Japan, China, India, Iran, Tunisia, Estonia, Turkey, Vietnam, and Europe, most of which used to be developing regions with a predominance of consanguineous marriage [6-15]. In the present study, we report two novel *TACSTD2* mutations from 3 Japanese GDLD patients.

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METHODS

Ethical issues: All experimental procedures were approved by the Institutional Review Board for Human Studies at Kyoto Prefectural University of Medicine, Kyoto, Japan. Prior informed consent was obtained from all patients after a detailed explanation of the study protocols, and this study was performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

Subjects: All patients were given a complete ophthalmic examination including visual acuity testing, noncontact tonometry, and slit-lamp examination. For all 3 GDLD patients enrolled in this study, clinical diagnosis was confirmed based upon slit-lamp examination and the agreement of at least 2 corneal specialists in our department.

Sequencing analysis: Genomic DNA was extracted from peripheral blood using a commercially available column-based DNA extraction kit (DNeasy® Blood & Tissue Kit;

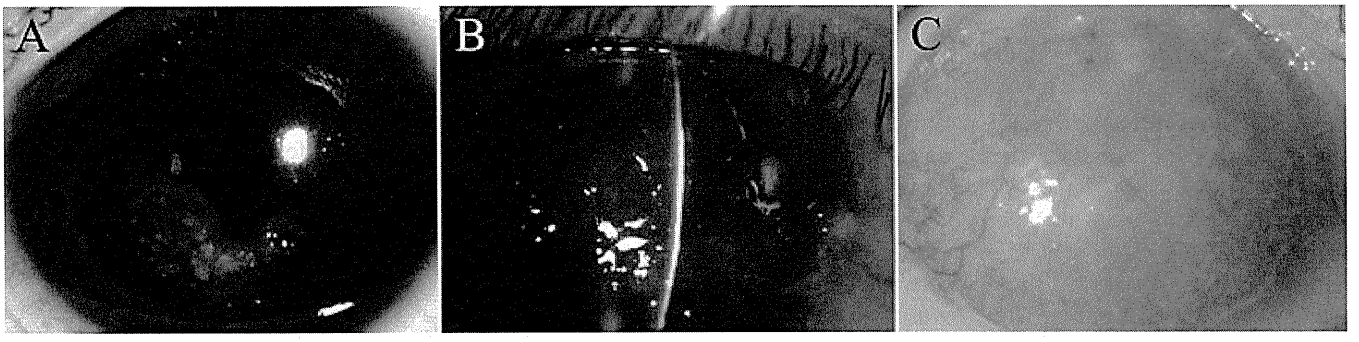

Figure 1. Images demonstrating the corneas of 3 unrelated GDLD patients. Proband A (**A**) and proband B (**B**) demonstrated mulberry-type GDLD corneas with multiple grayish subepithelial amyloid depositions. Proband C (**C**) demonstrated a kumquat-like GDLD cornea with neovascularization.

QIAGEN GmbH, Hilden, Germany). Sequencing analysis was performed using a commercially available kit (BigDye 3.1; Applied Biosystems, Inc., Foster City, CA). Polymerase chain reaction (PCR) was performed with a primer pair against *TACSTD2* (M1S1-F-2; 5′-CCT GCA GAC CAT CCC AGA C-3′, M1S1-R-2; 5′-CAG GAA GCG TGA CTC ACT TG-3′) which fully covered the coding sequence of this gene. The PCR product was bi-directionally sequenced in a 20-µl reaction buffer containing a 2× sequencing mixture and either of the above primers. After ethanol precipitation, the sequence products were electrophoresed on an automated capillary sequencer (Genetic Analyzer 3130xl; Applied Biosystems).

Validation of the sequencing data: As for the family members related to Case 1 and Case 2, sequencing data was validated by PCR using a primer pair (M1S1–20ins-F; 5′-TGA AGC GCC TCA CCG CCG GC-3′, M1S1–20ins-R; 5′-CGA CGA GGG CCA CCA CGA CC-3′) which encompass the site of the identified insertional mutation.

As for Case 3, sequencing data was validated by the single-base primer extension assay with a commercially available kit (SNaPshot® Multiplex System; Applied Biosystems) with a primer (SS-M1S1-Y225X: 5′-ATC GGC GAT GCC GCC TAC TA-3′).

Plasmid construction: For the protein expression of either the wild-type or mutated *TACSTD2*, DNA fragments covering an entire open reading frame with or without particular mutations were amplified by PCR, ligated into an expression vector pcDNA3.1/V5-His-TOPO (Invitrogen Corp., Carlsbad, CA), and transformed into chemically competent cells (JM109; TOYOBO Co., Ltd., Osaka, Japan). A single colony, which was confirmed via sequencing analysis to have the proper expected sequence without any unexpected mutations, was isolated, propagated, and subjected to the plasmid extraction using a commercially available column-based kit (NucleoBond; MACHEREY-NAGEL GmbH & Co., Düren, Germany).

Cell culture and gene transfer: SV40 immortalized human corneal epithelial (HCE-T) cells [16] were subcultured every 4 days and maintained in DMEM/F12 containing 200 U/ml penicillin and streptomycin, 10% fetal bovine serum (FBS; Cellgro; Mediatech, Inc., Herndon, VA), 0.1 µg/ml cholera toxin (List Biologic Laboratories, Inc., Campbell, CA), 5 µg/ml insulin (Sigma-Aldrich Corp., St. Louis, MO), and 10 ng/ml human epidermal growth factor (Invitrogen). After the cells had reached to 70%–80% confluency on a commercially available culture-glass slide (Nunc Lab-Tek™ Chamber Slide™ System; Thermo Fisher Scientific, Inc., Rochester, NY), each of the plasmids was transfected into the HCE-T cells using Lipofectamine™ LTX (Invitrogen) according to the manufacturer's instructions.

Immunocytostaining analysis: Cells grown on the culture-glass slide were fixed with Zamboni's fixative, blocked with 1% skim milk, and then incubated overnight with a primary antibody at 4 °C. The primary antibody included anti-V5 (MM IgG_1, clone V5005; NACALAI TESQUE, Inc., Kyoto, Japan) and normal mouse IgG_1 (Dako Denmark A/S, Glostrup, Denmark) as a negative control. After being washed with 0.01 M of phosphate buffered saline (PBS), the samples were incubated with a secondary antibody (Alexa Fluor® 488-labeled anti-mouse or anti-goat IgG; Invitrogen) at room temperature for 1 h. After being washed again with 0.01 M PBS, the sections or the cells were counter-stained with propidium iodide, mounted, covered with coverslips, and observed and photographed using a fluorescence microscope (AX70 TRF; Olympus Corporation, Tokyo, Japan).

RESULTS

Clinical findings: Case 1 involved a 30-year-old Japanese man (proband A) who had undergone photorefractive keratectomy (PTK) in his right eye at the age of 16 and in his left eye at the age of 21. His parents were second cousins to one-another. He had bilateral diffuse corneal opacities with multiple grayish-white nodular elevations located at the subepithelial region (Figure 1A) which fit the classification of typical mulberry GDLD [17].

Case 2 involved a 29-year-old Japanese female (proband B) who had undergone PTK in her left eye at the age of 23 and in her right eye at the age of 26. Her parents were first

cousins to one-another. Slit-lamp examination revealed grayish amyloid depositions in the bilateral corneas which fit the classification of typical mulberry GDLD (Figure 1B).

Case 3 involved an 83-year-old Japanese woman (proband C). Her parents' marriage was not consanguineous. She had undergone lamellar keratoplasty along with keratoepithelioplasty in her left eye at the age of 72 and penetrating keratoplasty in her right eye at the age of 82. Slit-lamp examination revealed the characteristic findings of a kumquat-like GDLD subtype with neovascularization in both of her eyes (Figure 1C). Recurrence of amyloid deposition was observed in both of her eyes. Surface keratectomy was performed for her left eye to remove the superficial amyloid depositions, but no surgical intervention was undertaken for her right eye because she was too elderly to undergo the operation at that time.

All surgeries for the 3 cases were performed to treat their GDLD corneas. After the surgeries, case 1 and case 2 continued to wear soft contact lenses and no recurrence was observed in the eyes of those patients, however, case 3 was unable to wear soft contact lenses and recurrence occurred in both of her eyes. Those findings are in good agreement with the previous study that reported the protective effect of using a soft contact lens for the postoperative GDLD cornea [18].

Mutation analysis: Sequencing analysis of *TACSTD2* revealed a homozygous, 20-base insertion mutation between the 840th and the 841st nucleotide positions (c. 840_841insTCATCATCGCCGGCCTCATC) for proband A and proband B (Figure 2C), resulting in a putative frameshift and a premature termination at the 303th amino acid position (p.Ile281SerfsX23). The respective parents of the proband A and proband B, as well as the younger sister of proband B, all of whom had no abnormal findings in their corneas, had one allele with a mutated *TACSTD2* gene and one allele with a wild-type *TACSTD2* gene (Figure 2E), indicating that the phenotype well co-segregates with the genotype in these pedigrees. Proband C was found to have a homozygous substitutive mutation from C to A at the 675th nucleotide position (c.675C>A), which may result in nonsense mutation at the 225th amino acid position (p.Tyr225X; Figure 2D). The sequence data were further validated by the difference in the length of the PCR products for proband A, proband B, and their respective family members or by the single-base primer extension analysis for proband C (Figure 2). Data for other family members related to proband C were not obtained due to the fact they refused permission to be enrolled in this study.

Subcellular localization of TACSTD2 protein: The V5-epitope tagged expression plasmid vector harboring either wild-type or mutated TACSTD2 protein was transfected into the HCE-T cells. Immunocytological staining analysis using anti-V5 antibody against the transfected HCE-T cells revealed that the normal TACSTD2 protein distributes both at the plasma membrane and in the cytoplasm while the mutated TACSTD2 protein was found to be diffusely distributed in the cytoplasm with no apparent plasma membrane localization (Figure 3). Detergent treatment with 0.1% Tween-20 significantly increased the number of V5-immunopositive cells among the cells transfected with the mutated *TACSTD2* gene, confirming the cytoplasmic localization of the mutated TACSTD2 protein.

DISCUSSION

In this study, we have identified two novel homozygous mutations from 3 unrelated GDLD patients with a phenotype well co-segregated with the genotype within their respective families. The insertional mutation of *TACSTD2* that was found in 2 of the GDLD patients may have resulted from a flame-shift amino acid alteration with premature termination (p.Ile281SerfsX23) within the transmembrane domain. A substitutive mutation found in 1 of the GDLD patients may have resulted from a nonsense mutation (p.Tyr225X) within a region between the thyroglobulin type-1 and transmembrane domains. The transmembrane domain should support the hydrophobic scaffold which may be fundamental to the membrane binding property of this protein. However, and as far as we know, such a domain structure is only a computationally speculated model from the primary amino acid structure of this protein. Therefore, the subcellular localization of both the wild-type and mutated TACSTD2 proteins was experimentally determined in this study.

Other than the changes in the subcellular localization of the TACSTD2 protein in the GDLD patients as identified in this current study, the functions of the TACSTD2 protein have yet to be elucidated. Using electron microscopy, Kinoshita et al. [19] demonstrated an enlarged intercellular space and facilitated scaling of the superficial cells of corneal epithelium in GDLD corneas. Quantock et al. [20] reported the increased permeability in the epithelium of the GDLD cornea using horseradish peroxidase as a molecular tracer. Takaoka et al. [21] found decreased expression of the tight junction–related protein including claudins (CLDNs), zonula occludens-1, and occludin in GDLD corneas. Recently, we discovered that the TACSTD2 protein directly binds to CLDN1 and 7 proteins and protects them from degradation by ubiquitin-proteasome system [22]. In the absence of functional TACSTD2 protein, the CLDN proteins will be degraded and tight junctions will not be formed, resulting in the hyperpermeation of tear fluid into the cornea, ultimately leading to the subepithelial deposition of amyloid in the cornea.

It has been reported that an AxxxG motif in the transmembrane domain of the epithelial cell adhesion molecule (EpCAM) protein, a paralogous gene of the *TACSTD2* gene, is involved in the binding of the EpCAM protein to the CLDN7 protein [23]. Since the transmembrane domain of the TACSTD2 protein also has the AxxxG motif at the corresponding site to the EpCAM protein [22], only the

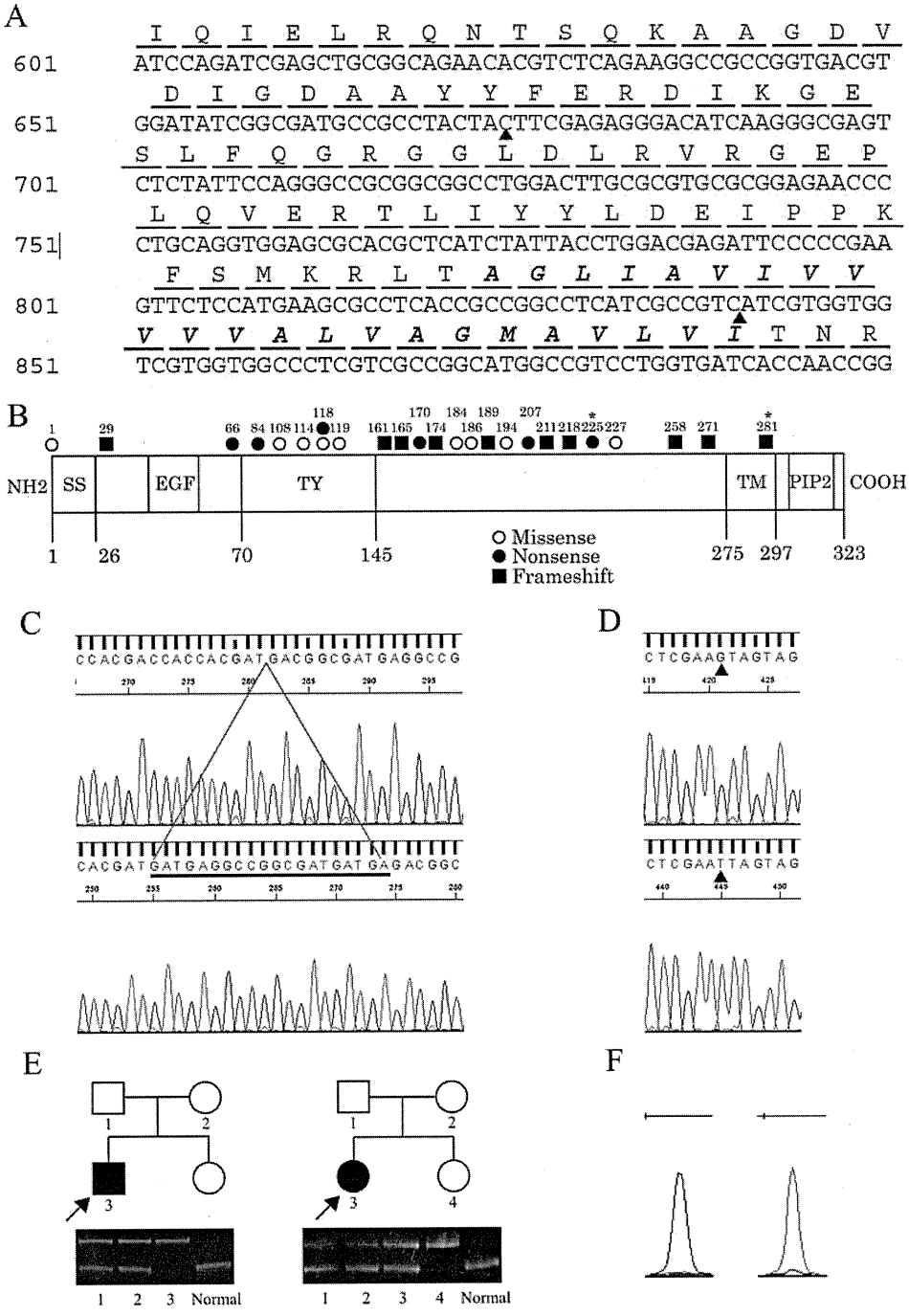

Figure 2. Results of sequencing analysis, PCR analysis, and single-base primer extension assay. **A**: Nucleotide and amino acid sequence of TACSTD2. Arrowheads indicate the site of the c.675C>A and c. 840_841insTCATCATCGCCGGCCTCATC nucleotide changes. Note that the amino acids in bold italic type are of the transmembrane domain. **B**: Computationally-predicted domain structure of the TACSTD2 protein with mutations of previous reports and this report (*). SS: signal sequence; EGF: EGF-like repeat; TY: thyroglobulin type I repeat; TM: transmembrane domain; PIP2: PIP2 binding sequence. **C**: Results of sequencing analysis of *TACSTD2* in a normal volunteer (upper) and in proband A or B (lower). The underlined nucleotides indicate the inserted 20-base sequence between the 840th and 841st nucleotide positions of *TACSTD2*. Note that the presented sequence is in a reverse direction. **D**: Results of sequencing analysis of *TACSTD2* in a normal volunteer (upper) and in proband C (lower). Arrowheads indicate the site of the c.675C>A mutation. Note that the presented sequence is in a reverse direction. **E**: Results of PCR analysis to examine the difference in length between the normal and insertion-bearing alleles in the families of the proband A (left) and proband B (right). The upper bands indicate the PCR product derived from the insertion-bearing alleles while the lower bands indicate the PCR product from the normal alleles. Note that the sister of proband A was not examined. **F**: Results of 1-base primer extension analysis for the 675th nucleotide of *TACSTD2* in the normal volunteer (left) and the proband C (right). Black indicates C and green indicates A. Note that the presented data was produced by the forward primer.

membrane-bound TACSTD2 protein seems to have the potential to execute the binding activity to CLDNs. Thus, we strongly believe that the mutated TACSTD2 protein being devoid of the binding property to the plasma membrane is actually pathological, as is shown in the present study.

Interestingly, the 20-base insertion mutation was found in 2 unrelated GDLD patients. Considering the fact that this mutation has thus-far not been reported, along with the fact that insertion mutations tend to be much rarer compared to substitution mutations, this mutation seems to be a founder mutation caused in a single Japanese ancestor, as has been reported in GDLD [6] and TGFBI-related corneal dystrophies [24-26]. Therefore, although these 2 GDLD patients are not related to one-another, they may have a common ancestor who may bear one de novo mutation of *TACSTD2*, possibly at one of his or her alleles.

In summary, we report here two novel mutations of *TACSTD2* and their altered subcellular localization in the