TABLE 2. Agreements Reached in the Definition/Diagnosis Panel

- The following findings are mandatory to diagnose keratoconus
  - Abnormal posterior elevation
  - Abnormal corneal thickness distribution
  - Clinical noninflammatory corneal thinning
- Keratoconus and PMD are different clinical presentations of the same disease
- The aspect that distinguishes keratoconus, PMD, and keratoglobus is "thinning location and pattern"
- Keratoconus and PMD are best differentiated by a combination of
  - Full tomographic corneal thickness map
  - Slit-lamp examination
  - Anterior curvature map
  - Anterior tomographic elevation map
- As opposed to "thinning disorders" the following are classified under "ectatic diseases"
  - Keratoconus
  - PMD
  - Keratoglobus
  - Postrefractive surgery progressive corneal ectasia
- Keratoglobus and keratoconus are different clinical entities
- True unilateral keratoconus does not exist
- The best current and widely available diagnostic test to diagnose early keratoconus is tomography (Scheimpflug or optical coherence tomography)
- Currently, there is no clinically adequate classification system for keratoconus
- Posterior corneal elevation abnormalities must be present to diagnose early or subclinical keratoconus
- Secondary induced ectasia may be caused by a pure mechanical process (and can be unilateral)
- Central pachymetry is the least reliable indicator (determinant) for diagnosing keratoconus
- The pathophysiology of keratoconus is likely to include the following components
  - Genetic disorder
  - Biochemical disorder
  - Biomechanical disorder
  - Environmental disorder
- Placido-based topography analyzes the central anterior corneal surface, whereas tomography (Scheimpflug and/or optical coherence tomography) analyzes the anterior and posterior cornea and produces a near full corneal thickness map
- Keratoconus can be present in a cornea of normal central thickness
- Ectasia progression is defined by a consistent change in at least 2 of the following parameters where the magnitude of the change is above the normal noise of the testing system
  - Progressive steepening of the anterior corneal surface
  - Progressive steepening of the posterior corneal surface
  - Progressive thinning and/or an increase in the rate of corneal thickness change from the periphery to the thinnest point
- The changes need to be consistent over time and above the normal | variability (ie, noise) of the measurement system (this will vary by system). Although progression is often accompanied by a decrease in BSCVA, a change in both uncorrected visual acuity and BSCVA is not required to document progression
- Risk factors for keratoconus: Down syndrome, relatives of affected patients especially if they are young, ocular allergy, ethnic factors (Asian and Arabian), mechanical factors, eg, eye rubbing, floppy eyelid syndrome, atopy, connective tissue disorders (Marfan syndrome), Ehlers–Danlos syndrome and Leber congenital amaurosis

(there is evidence that preservatives are associated with irritation, eye rubbing, and epithelial microtrauma).[42–44]

Regarding refraction and optical correction, subjective refraction should be attempted in all patients with ectasia. In addition, aberrometry may help to determine the optical correction in early disease. Progressive addition glasses are not contraindicated in eyes with keratoconus or other ectasias, but they are rarely successful.

The use of contact lenses in patients with keratoconus and other ectasias was extensively debated during the 2 first rounds and the face-to-face meeting. The group recognized their importance for visual rehabilitation and agreed that their use does not slow or halt progression of corneal ectasias. Still, the use of contact lenses for purely cosmetic reasons should be discouraged in this group of patients because of the difficulty in contact lens fitting and the increased risk of complications from a poorly fit contact lens.

Rigid contact lenses should be used in cases of unsatisfactory vision with glasses or conventional soft contact lenses. Among the rigid contact lenses, gas-permeable lenses are preferred and should be tried initially in patients with keratoconus. Moreover, in a patient with keratoconus who has failed a trial of conventional corneal rigid gas-permeable lenses, the alternative contact lens options would be: hybrid lens (rigid center, soft skirt); toric, bitoric, and keratoconus design soft contact lens; keratoconus design corneal rigid gas-permeable contact lens; piggy-back; corneoscleral, miniscleral, and semiscleral contact lens; and scleral lens.

During the face-to-face discussion, the group felt that it was important to identify special situations where keratoconus evaluation should be considered/recommended. A careful evaluation is strongly recommended in patients with Down syndrome and should be considered in patients with known risk factors for developing keratoconus (see Definition/diagnosis risk factors above). The panel also agreed that pregnancy could contribute to acceleration of the progression of ectasia.

It was also agreed that in acute hydrops, nonsurgical or less invasive surgical management such as intracameral gas injection should be attempted before keratoplasty. The consensuses reached by the Nonsurgical management panel are summarized in Table 3.

Surgical Management

In the first 2 rounds and during the face-to-face discussion of surgical management, the question of when to proceed to surgery was debated. Overall, experts have good access to experienced practitioners or experts in contact lens fitting, inside or outside their institution. The consensus was that surgery should be considered when patients were not fully satisfied with nonsurgical treatments. In general, panelists preferred the term *"satisfactory best-corrected"* rather than *"best-corrected"* vision because it differentiates patients who may be able to achieve good corrected vision, for example, with lenses, but are unable to tolerate them or wear them comfortably for long periods of time.

CXL is currently available and is performed by the majority of the panelists (83.3%) for keratoconus, using

TABLE 3. Agreements Reached in the Nonsurgical Management Panel

Statements
• The 2 most important goals of management are halting disease progression and visual rehabilitation. Verbal guidance should be given to patients regarding the importance of not rubbing one's eyes, use of topical antiallergic medication in patients with allergy, and use of topical lubricants (in the case of ocular irritation) to decrease the impulse to eye rub
• In cases of allergy or if there is any allergic component, patients should be treated with topical antiallergic medication and lubricants. Topical multiple-action antiallergic medications (ie, antihistamines, mast cell stabilizer, antiinflammatory) should be used in patients with keratoconus with atopy or history of eye rubbing
• There is no direct relationship between keratoconus and dry eye
• Preservative-free agents are preferred as they are associated with less irritation and epithelial trauma compared with agents with preservatives
• Subjective refraction should be attempted in all patients with corneal ectasia. Aberrometry may help to determine the optical correction in early disease
• Progressive-type glasses are not contraindicated in eyes with keratoconus or other ectasias, but they are rarely successful
• Contact and scleral lenses are extremely important for visual rehabilitation in patients with keratoconus and other corneal ectasias
• Contact lens use does not slow or halt progression of corneal ectasias
• Rigid contact lenses should be used in cases of unsatisfactory vision with glasses or conventional soft contact lenses. Among the rigid contact lenses, gas-permeable lenses are preferred and should be tried initially in patients with keratoconus. In a patient with keratoconus who has failed conventional corneal gas-permeable lenses, alternative contact lens options would be: hybrid lens (rigid center, soft skirt); toric, bitoric, and keratoconus design soft contact lens; keratoconus design corneal rigid gas-permeable contact lens; piggy-back; corneoscleral, miniscleral, and semiscleral contact lens; and scleral lens
• A careful evaluation for keratoconus is strongly recommended in patients with Down syndrome and should be considered in patients with known risk factors for developing keratoconus (Table 2)
• Pregnancy could contribute to acceleration of the progression of ectasia
• In acute hydrops, nonsurgical management should be attempted before keratoplasty

a variety of techniques. The panelists who do not have current access to CXL were willing to use this technique once it becomes available. In addition, it was recognized that *the term "collagen cross-linking"* is not currently considered correct and should be replaced by *"corneal cross-linking."*

Regarding the indication for CXL, the panelists found that CXL is extremely important in the treatment of keratoconus with documented clinical progression; it is very important for the treatment of postrefractive surgery keratectasia; it is important for the treatment of keratoconus with a perceived risk of progression (ie, clinical progression has not been confirmed) and for eyes with keratoconus that have previously received other forms of corneal surgery (such as ICRS or PRK). There was no consensus about the use of CXL in subclinical keratoconus. The surgical management of keratoglobus is typically quite different from keratoconus and was not considered in the treatment questionnaires.

In terms of restrictions for CXL, the panelists agreed that there is no age below or above which CXL should be restricted in keratoconic eyes with evidence of progression. As for keratoconic eyes without evidence of progression, there was no consensus on whether there is an age below which CXL should be restricted, but it is rarely indicated in patients older than 40 years. There was no consensus on an uncorrected vision better than which CXL should be restricted in either keratoconic eyes with or without evidence of progression.

Besides CXL, anterior lamellar keratoplasty (ALK), more specifically descemetic deep ALK (dDALK), and penetrating keratoplasty (PK) are the most frequent surgical modalities used in the surgical treatment of keratoconus. ICRS are also used, but to a lesser degree. However, superficial keratectomy (manual or PTK), PRK, conductive keratoplasty, incisional keratotomy (arcuate/parallel incisions), microwave corneal remodeling, and clear lens extraction with spherical/toric IOL are uncommonly used by the expert panel.

Experts who currently use DALK agreed that the most important patient-related factor in determining the need for this type of surgery is contact lens intolerance. As for PK, the most important factor in considering keratoplasty in keratoconus is when significant corneal scarring (eg, posthydrops) is present. Other important factors included the following: the patient is contact lens intolerant or is not keen on wearing contact lenses; other surgical strategies fail, or are contraindicated; the cornea is very thin (<200 μm); and the keratoconus is deemed to be severe and at a potential risk of acute hydrops. In this context, there was no consensus regarding the importance of apparent rapid progression of keratoconus.

According to the expert panel, any form of corneal transplant is offered to 21% to 60% of patients with keratoconus who are eligible for surgery. Among all keratoplasties, some form of ALK (at least attempted) is currently performed in more than 60% of patients. In the absence of previous hydrops (ie, no previous compromise of Descemet membrane), some form of ALK (at least attempted) is performed in more than 60% of the patients, whereas in cases with previous hydrops and deep scarring (ie, previous compromise of Descemet membrane), some form of ALK is performed (at least attempted) in 0% to 20% of patients.

Regarding ALK techniques used by the panelists in keratoconus with no previous evidence of acute hydrops, dDALK with big bubble technique is the most common technique (more than 51% of the cases). Microkeratome-assisted ALK is never performed and other ALK techniques such as manual layer-by-layer predescemetic DALK (pdDALK), dDALK with viscodissection, pdDALK with the Melles technique, and femtosecond laser-assisted DALK are performed in less than 25% of the patients. Regarding the ALK techniques used by the panelists in keratoconus with previous evidence of acute hydrops, microkeratome-assisted ALK is never performed and the remaining techniques are performed in less than 25% of the patients.

Although half of the panel have performed femtosecond laser-assisted PK for keratoconus, the majority of PKs are performed with a standard (nonlaser) technique. Of those surgeons performing femtosecond laser-assisted surgery, the percentage of cases varies from 1% to 20%.

The panelists concluded that the most important surgical techniques to restore the best uncorrected visual acuity possible in keratoconus are (in the order of importance): dDALK, PK, and ICRS. The most important surgical techniques to restore the best rigid gas-permeable contact lens

(RGP-CL) corrected visual acuity possible are (in the order of importance): dDALK and PK.

The most common approaches were pooled and are presented in Table 4, which describes which treatment practices are considered in specific case scenarios in which age, stage of disease, and visual acuity are varying factors. A flowchart describing a logical management sequence for a patient with keratoconus is presented in Figure 2.

DISCUSSION

Based on the literature and positive previous experience with dry eye, allergy, and infection prophylaxis, we chose a modified Delphi technique to achieve consensus regarding the most important topics in keratoconus and other corneal ectasias.[32,36,38] Before this project, we undertook a successful pilot of this specific consensus with Latin-American corneal specialists as a test-run that was approved by the 4 cornea societies.[45] One of the advantages of the Delphi method is that information can be gathered from a geographically diverse panel of participants while keeping their anonymity, which reduces the halo effects associated with the opinions of prominent participants.[46,47] It also allows the panelists adequate time to carefully consider their responses before replying.[46,47] The reliability of this method increases with the number of participants and rounds.

To achieve a global representation of experts in corneal ectasias, we decided that all 4 of the main active recognized supranational cornea societies would be responsible for the selection of coordinators (9) and panelists (36). This panel size is in line with most Delphi studies and assured sufficient worldwide expertise, even if attrition occurred.[40] The international representation of the panel strengthens our findings, reflecting a broad range of clinical opinions from diverse geographical regions of the world and a variety of clinical practices.

We achieved a 100% response rate in the first 2 rounds in all 3 panels. Possible reasons for the high response rate achieved could be attributed to the high motivation from the panel of experts who recognized the relevance of the project. In addition, the quick turnaround time, the clear time frame, and the personalized reminders might have also contributed to these high rates. The number of panelists who attended the face-to-face meeting in Chicago and responded to the third round questionnaire was somewhat smaller (29/36 or 80.5%). Considering the logistical difficulties for some of the panelists from outside the United States and the lack of direct funding, we thought it was an excellent attendance.

Although extensively used in the health and technology fields, Delphi and other consensus methods have some limitations. Delphi can pose some difficulties in keeping the interest of the panelists after 2 or more rounds and the costs involving each additional round. Also, if personal contact among participants is desirable, then Delphi is not appropriate. That was the reason we decided to use the modified Delphi method that included a third face-to-face round. Sackman, in his critical analysis of conventional Delphi, pointed out other limitations including the possibility of a crude questionnaire design, vulnerability with respect to who is an "expert," and obliviousness to reliability measurement and scientific validation of findings.[46,48] Despite these limitations, we found that the modified Delphi was the best technique for this project. The fact that it was funded by a grant from the Asia Cornea Foundation, without the participation of any company with a possible conflict of interest in the topic, strengthens the importance of this consensus and makes it even more representative of what cornea specialists think about keratoconus and corneal ectasias today.

TABLE 4. Panel Consensus to Surgical Approaches Based on Different Case Scenarios

• Young (eg, 15-year-old) patient with stable KCN with satisfactory vision with glasses
Prescribe glasses only or in combination with contact lenses or CXL
• Young (eg, 15-year-old) patient with progressive KCN with satisfactory vision with glasses
Perform CXL and prescribe glasses ± contact lenses
• Older (eg, 55-year-old) patient with stable KCN with satisfactory vision with glasses
Prescribe glasses only or with contact lenses
• Older (eg, 55-year-old) patient with progressive KCN with satisfactory vision with glasses?
Perform corneal cross-linking only or with prescription of glasses/ contact lenses
• Patient with stable KCN with unsatisfactory vision with glasses but satisfactory vision with rigid contact lenses and tolerates them well? This patient has a spherical equivalent of moderate myopia [eg, −5 diopters (D)]
Prescribe contact lenses (including scleral lenses)
• Patient with stable KCN with unsatisfactory vision with glasses but good vision with rigid contact lenses, and tolerates them well? This patient has a spherical equivalent of high myopia (eg, −15 D)
Prescribe contact lenses (including scleral lenses)
• Patient with stable KCN with unsatisfactory vision with glasses and contact and scleral lenses, or who does not tolerate contact or scleral lenses? This patient has a spherical equivalent of moderate myopia (eg, −5 D)
Perform dDALK. Consider ICRS in eyes with adequate corneal thickness and minimal to no scarring
• Patient with stable KCN with unsatisfactory vision with glasses and contact and scleral lenses, or who does not tolerate contact or scleral lenses? This patient has a spherical equivalent of high myopia (eg, −15 D)
Perform dDALK
• Patient with stable severe KCN with unsatisfactory vision with glasses and contact and scleral lenses? This patient has moderate anterior corneal scarring but no evidence of previous corneal hydrops
Perform dDALK
• Patient with stable severe KCN with unsatisfactory vision with glasses and contact and scleral lenses? This patient has moderate anterior and deep corneal scarring with evidence of previous corneal hydrops
PK alone or attempt pdDALK

KCN indicates keratoconus.

Definition/Diagnosis

The last decade has seen a dramatic change in the management of ectatic disease. Newer treatment modalities such as CXL have moved the timing of intervention to much earlier in the disease process. No longer are we delaying invasive treatments until there is significant loss of vision. Earlier intervention, however, imposes greater diagnostic challenges, as accurately identifying early ectatic change is

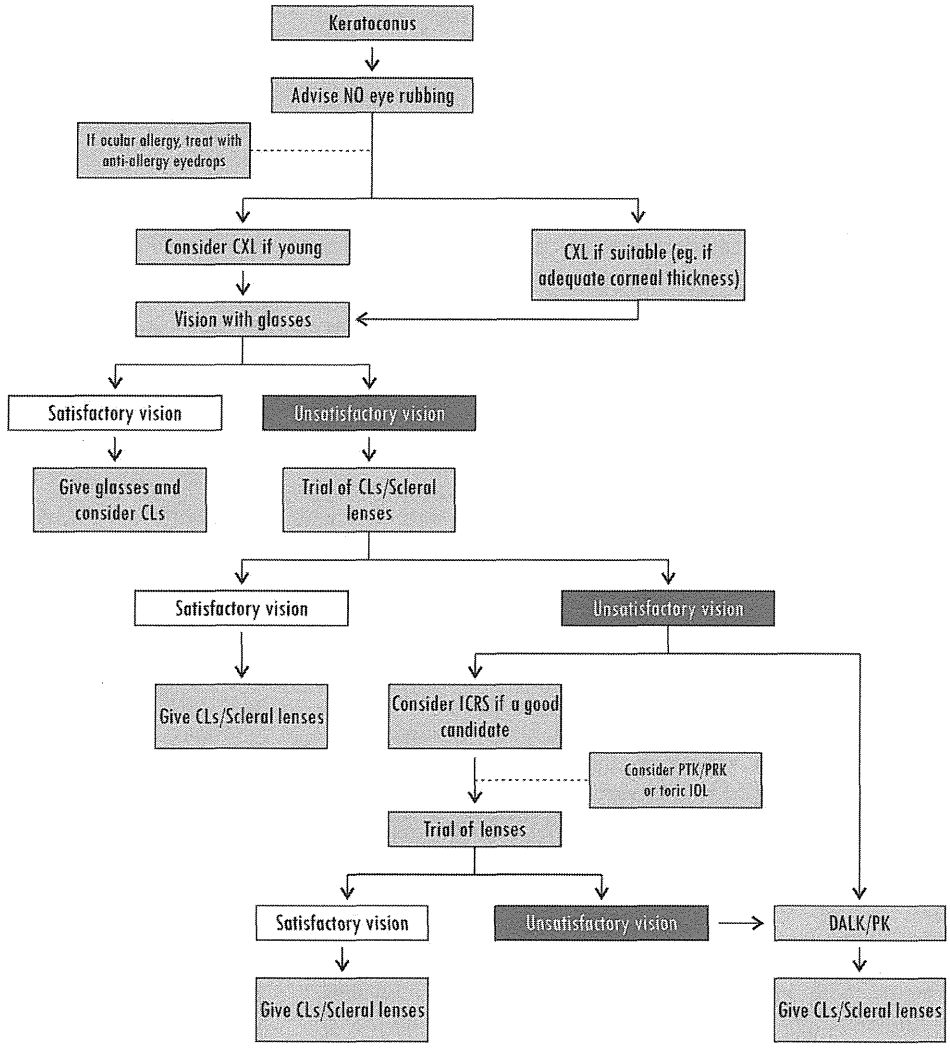

FIGURE 2. Keratoconus treatment flowchart. CLs, contact lenses; CXL, corneal cross-linking; PTK, phototherapeutic keratectomy.

more problematic than the identification of moderate to advanced disease. These greater diagnostic demands have fortunately been accompanied by significant improvements in corneal imaging with the emergence of both Scheimpflug imaging and optical coherence tomography. These devices can measure both anterior and posterior corneal surfaces, produce a corneal thickness map, and reconstruct the anterior segment. This advanced imaging is called corneal tomography to separate it from Placido disc–based videokeratographs that can only image the anterior corneal surface (topography).

The panel acknowledged the limitations of the often used, but dated, keratoconus classifications/staging systems [both Amsler–Krumeich[41] and CLEK (Collaborative Longitudinal Evaluation of Keratoconus[49])]. And, while the group recognized tomography as a critical diagnostic component, the panel also agreed that a suitable classification system using this additional information currently does not exist. Therefore, studies that correlate clinical findings such as visual performance (ie, BSCVA) with corneal topometric and tomographic parameters are needed. Additionally, the group agreed that documenting ectasia progression requires changes in at least 2 of the following; steepening of the anterior surface, steepening of the posterior surface, and/or thinning or changes in the pachymetric rate of change. Although these changes were noted as a requisite for documenting progression, the absolute magnitude of the changes is currently unknown. It was recognized, however, that younger patients should be examined for change at shorter time intervals as ectatic change can progress rapidly in this group.

The emergence of corneal/anterior segment tomography and the realization of the importance of the posterior cornea as an early indicator of ectatic change are reflected in the expert panel's opinion that both changes on the posterior corneal surface and alteration in the corneal thickness progression are necessary to diagnose keratoconus. Additionally, the importance of tomography is reflected in the group's view that the corneal thickness map, in addition to slit-lamp examination and anterior measurements, is necessary to properly differentiate PMD from keratoconus.

Other areas of consensus were that keratoconus and PMD are different clinical presentations of the same basic disease process and that the term "ectatic" diseases should be reserved for keratoconus, PMD, postrefractive surgery ectasia, and keratoglobus. Other "thinning" conditions, such as Terrien marginal degeneration, dellen, rheumatoid/autoimmune melts, etc, should be classified under the general term "corneal thinning disorders."

Finally, the pathophysiology of keratoconus was discussed. Keratoconus (and other ectatic disorders) was recognized as a multifactorial disease with genetic, biochemical, biomechanical, and environmental components. And, although it was felt that true unilateral keratoconus does not exist, it was appreciated that a unilateral clinical presentation may occur in a predisposed individual because of asymmetric environmental factors, such as eye rubbing. The findings are summarized in Table 2.

Nonsurgical Treatment

Corneal ectasias can be treated by nonsurgical approaches. Usually used in the initial stages, this form of treatment is often very successful. It is crucial to first define the goals for these less invasive therapeutic strategies. The panelists found that the most important objective of nonsurgical treatment is to halt progression; the second one is visual rehabilitation. Of course, these 2 goals are related and might be extrapolated to the surgical management as well. But together they represent the most important goals for successful treatment of corneal ectasia by ophthalmologists.

The most important nonsurgical treatment measures were patients' verbal guidance regarding the importance of not rubbing one's eyes. There is no evidence that a particular medication can halt the progression of ectatic corneal diseases. The majority of this research focuses on new antiinflammatory molecules or innovative technologies to induce transepithelial CXL.[50,51] It is possible that in the future, the researchers will find a topical medication that could directly influence the progression of keratoconus and other corneal ectasias.

The use of contact lenses in patients with keratoconus was extensively explored. The panelists agreed that although extremely beneficial to correct vision in a many patients, it does not slow or halt the progression of ectasia.[52] Rigid contact lenses should be tried first in patients with keratoconus. Numerous alternative contact and scleral lens options are available. The options varied according to regional access to some of these lenses and whether the corneal panelists do or do not fit contact lenses in their practices. The findings are summarized in Table 3.

Surgical Treatment

Determining the best surgical approach for keratoconus and other ectasias turned out to be a difficult task for a variety of reasons. For one, there are a large number of surgical procedures that are used to treat these conditions, some quite frequently and others much less frequently, and we included essentially all of these options as possible answers for the panelists. The wide geographic distribution of the panelists and the fact that some surgical options are more readily available in some countries than others made achieving a consensus difficult. Additionally, just keratoconus (not to mention the other ectasias) comes in a wide range of severity. The irregular astigmatism may be mild to severe. The corneal thinning may be mild to severe. There may or may not be associated high myopia. There may be severe scarring or a history of acute hydrops. In the end, we felt that it was most useful to present a wide variety of patient scenarios attempting to encompass the majority of patients with keratoconus we encounter in clinical practice and see whether we could get a consensus on management of these specific patients.

As a rule, the panelists felt that anyone with progressive ectasia should undergo CXL no matter what age or level of vision (assuming the eye was an appropriate candidate). Panelists also felt it was best not to proceed with surgery (other than CXL) if patients were satisfied with their vision with glasses or contact lenses. ICRS were routinely performed by some panelists and rarely or never performed by other panelists. The situation with phakic IOLs was similar, although they were performed less commonly than ICRS. There was a strong preference for DALK when a corneal transplant was needed, unless the eye had previous compromise of Descemet membrane (most commonly from acute hydrops), at which point the preference was for a PK. A minority of panelists strongly preferred pdDALK even in the presence of previous hydrops. The findings are summarized in Table 4.

CONCLUSIONS

Practice patterns in medicine certainly vary throughout the world. However, with increased international travel and improved communications, among other reasons, these differences seem to be diminishing. This global consensus using a modified Delphi technique resulted in definitions, statements, and recommendations for the diagnosis and management of keratoconus and other ectatic diseases. It should help eye care providers around the world to adopt best practices for these often visually debilitating conditions.

ACKNOWLEDGMENTS

The authors acknowledge the contribution of the Executive Directors of all the Cornea Societies involved: Gail J. Reggio (Cornea Society), Pang Chia Li (Asia Cornea Society), Karen Hodous (PanCornea), and Lorraine O'Hagan (EuCornea).

REFERENCES

1. Krachmer JH, Feder RS, Belin MW. Keratoconus and related noninflammatory corneal thinning disorders. *Surv Ophthalmol.* 1984;28:293–322.
2. Rabinowitz YS. Keratoconus. *Surv Ophthalmol.* 1998;42:297–319.
3. Belin MW, Villavicencio OF, Ambrosio R Jr. Tomographic parameters for the detection of keratoconus: suggestions for screening and treatment parameters. *Eye Contact Lens.* 2014;40:326–330.
4. Kennedy RH, Bourne WM, Dyer JA. A 48-year clinical and epidemiologic study of keratoconus. *Am J Ophthalmol.* 1986;101:267–273.

5. Jonas JB, Nangia V, Matin A, et al. Prevalence and associations of keratoconus in rural maharashtra in central India: the central India eye and medical study. *Am J Ophthalmol.* 2009;148:760–765.
6. Gokhale NS. Epidemiology of keratoconus. *Indian J Ophthalmol.* 2013; 61:382–383.
7. Faria-Correia F, Luz A, Ambrósio R Jr. Managing corneal ectasia prior to keratoplasty. *Expert Rev Ophthalmol.* 2015;10:33–48.
8. Kwitko S, Severo NS. Ferrara intracorneal ring segments for keratoconus. *J Cataract Refract Surg.* 2004;30:812–820.
9. Pena-Garcia P, Alio JL, Vega-Estrada A, et al. Internal, corneal, and refractive astigmatism as prognostic factors for intrastromal corneal ring segment implantation in mild to moderate keratoconus. *J Cataract Refract Surg.* 2014;40:1633–1644.
10. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol.* 2003;135:620–627.
11. Vinciguerra P, Randleman JB, Romano V, et al. Transepithelial iontophoresis corneal collagen cross-linking for progressive keratoconus: initial clinical outcomes. *J Refract Surg.* 2014;30:746–753.
12. Koller T, Pajic B, Vinciguerra P, et al. Flattening of the cornea after collagen crosslinking for keratoconus. *J Cataract Refract Surg.* 2011;37: 1488–1492.
13. Kymionis GD, Grentzelos MA, Kankariya VP, et al. Long-term results of combined transepithelial phototherapeutic keratectomy and corneal collagen crosslinking for keratoconus: cretan protocol. *J Cataract Refract Surg.* 2014;40:1439–1445.
14. Koller T, Iseli HP, Donitzky C, et al. Topography-guided surface ablation for forme fruste keratoconus. *Ophthalmology.* 2006;113:2198–2202.
15. Hashemian SJ, Soleimani M, Foroutan A, et al. Toric implantable collamer lens for high myopic astigmatism in keratoconic patients after six months. *Clin Exp Optom.* 2013;96:225–232.
16. Shafik Shaheen M, El-Kateb M, El-Samadouny MA, et al. Evaluation of a toric implantable collamer lens after corneal collagen crosslinking in treatment of early-stage keratoconus: 3-year follow-up. *Cornea.* 2014;33: 475–480.
17. Kymionis GD, Grentzelos MA, Karavitaki AE, et al. Combined corneal collagen cross-linking and posterior chamber toric implantable collamer lens implantation for keratoconus. *Ophthalmic Surg Lasers Imaging.* 2011;42:e22–e25.
18. Kanellopoulos AJ, Binder PS. Management of corneal ectasia after LASIK with combined, same-day, topography-guided partial transepithelial PRK and collagen cross-linking: the athens protocol. *J Refract Surg.* 2011;27:323–331.
19. Kanellopoulos AJ. Comparison of sequential vs same-day simultaneous collagen cross-linking and topography-guided PRK for treatment of keratoconus. *J Refract Surg.* 2009;25:S812–S818.
20. Tan DT, Anshu A, Parthasarathy A, et al. Visual acuity outcomes after deep anterior lamellar keratoplasty: a case-control study. *Br J Ophthalmol.* 2010;94:1295–1299.
21. Amayem AF, Hamdi IM, Hamdi MM. Refractive and visual outcomes of penetrating keratoplasty versus deep anterior lamellar keratoplasty with hydrodissection for treatment of keratoconus. *Cornea.* 2013;32:e2–e5.
22. Buzzonetti L, Petrocelli G, Valente P. Big-bubble deep anterior lamellar keratoplasty assisted by femtosecond laser in children. *Cornea.* 2012;31: 1083–1086.
23. Kook D, Derhartunian V, Bug R, et al. Top-hat shaped corneal trephination for penetrating keratoplasty using the femtosecond laser: a histomorphological study. *Cornea.* 2009;28:795–800.
24. Buratto L, Bohm E. The use of the femtosecond laser in penetrating keratoplasty. *Am J Ophthalmol.* 2007;143:737–742.
25. Jones J, Hunter D. Consensus methods for medical and health services research. *BMJ.* 1995;311:376–380.
26. Coia LR, Minsky BD, John MJ, et al. Patterns of care study decision tree and management guidelines for esophageal cancer. American College of Radiology. *Radiat Med.* 1998;16:321–327.
27. Pelton JN. The future of telecommunications: a Delphi survey. *J Commun.* 1981;31:177–189.
28. Holmes ER, Tipton DJ, Desselle SP. The impact of the internet on community pharmacy practice: a comparison of a Delphi panel's forecast with emerging trends. *Health Mark Q.* 2002;20:3–29.
29. Darling G, Malthaner R, Dickie J, et al; Lung Cancer Surgery Expert Panel. Quality indicators for non-small cell lung cancer operations with use of a modified Delphi consensus process. *Ann Thorac Surg.* 2014;98:183–190.
30. Richter A, Ostrowski C, Dombeck MP, et al. Delphi panel study of current hypertension treatment patterns. *Clin Ther.* 2001;23:160–167.
31. Young LJ, George J. Do guidelines improve the process and outcomes of care in delirium? *Age Ageing.* 2003;32:525–528.
32. Behrens A, Doyle JJ, Stern L, et al. Dysfunctional tear syndrome: a Delphi approach to treatment recommendations. *Cornea.* 2006;25:900–907.
33. Ma Quintana J, Escobar A, Bilbao A. Explicit criteria for prioritization of cataract surgery. *BMC Health Serv Res.* 2006;6:24.
34. Lee PP, Sultan MB, Grunden JW, et al. Assessing the Importance of IOP Variables in Glaucoma Using a Modified Delphi Process. *J Glaucoma.* 2010;19:281–287.
35. Douglas RS, Tsirbas A, Gordon M, et al. Development of criteria for evaluating clinical response in thyroid eye disease using a modified Delphi technique. *Arch Ophthalmol.* 2009;127:1155–1160.
36. Abad JC, Gomes JAP, Guzzo G, et al. Latin American consensus on antimicrobial prophylaxis in ocular surgery. *J Cataract Refract Surg.* 2010;36:865–866.
37. Ferris FL III, Wilkinson CP, Bird A, et al. Clinical classification of age-related macular degeneration. *Ophthalmology.* 2013;120:844–851.
38. Santos MS, Alves MR, Freitas D, et al. Ocular allergy Latin American consensus. *Arq Bras Oftalmol.* 2011;74:452–456.
39. Fitch K, Bernstein SJ, Aguilar MS, et al. *The RAND/UCLA Appropriateness Method User's Manual.* Santa Monica, CA: RAND; 2001.
40. Ambrosio R Jr, Caiado AL, Guerra FP, et al. Novel pachymetric parameters based on corneal tomography for diagnosing keratoconus. *J Refract Surg.* 2011;27:753–758.
41. Krumeich JH, Daniel J, Knulle A. Live-epikeratophakia for keratoconus. *J Cataract Refract Surg.* 1998;24:456–463.
42. Baudouin C, Labbé A, Liang H, et al. Preservatives in eyedrops: the good, the bad and the ugly. *Prog Retin Eye Res.* 2010;29:312–334.
43. Chen W, Li Z, Hu J, et al. Corneal alterations induced by topical application of benzalkonium chloride in rabbit. *PLoS One.* 2011;6:e26103.
44. Jee D, Park SH, Kim MS, et al. Antioxidant and inflammatory cytokine in tears of patients with dry eye syndrome treated with preservative-free versus preserved eye drops. *Invest Ophthalmol Vis Sci.* 2014;55:5081–5089.
45. Ambrósio R, Belin MW, Perez VL, et al. Definitions and concepts on keratoconus and ectatic corneal diseases: Panamerican Delphi Consensus —A Pilot for the Global Consensus on Ectasias. *Int J Kerat Ect Cor Dis* 2014;3. in press.
46. Fink A, Kosecoff J, Chassin M, et al. Consensus methods: characteristics and guidelines for use. *Am J Public Health.* 1984;74:979–983.
47. Gordon TJ. *The Delphi Method.* Washington, DC: American Council for the United Nations University; 1994.
48. Sackman H. *Delphi Critique.* Lexington, MA: D.C. Health; 1975.
49. McMahon TT, Szczotka-Flynn L, Barr JT, et al. A new method for grading the severity of keratoconus: the Keratoconus Severity Score (KSS). *Cornea.* 2006;25:794–800.
50. Bottos KM, Oliveira AG, Bersanetti PA, et al. Corneal absorption of a new riboflavin-nanostructured system for transepithelial collagen cross-linking. *PLoS one.* 2013;8:e66408.
51. Kim M, Takaoka A, Hoang QV, et al. Pharmacologic alternatives to riboflavin photochemical corneal cross-linking: a comparison study of cell toxicity thresholds. *Invest Ophthalmol Vis Sci.* 2014;55: 3247–3257.
52. Barr JT, Wilson BS, Gordon MO, et al. Estimation of the incidence and factors predictive of corneal scarring in the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) Study. *Cornea.* 2006;25:16–25.

Jpn J Ophthalmol
DOI 10.1007/s10384-015-0408-y

THE OFFICIAL INTERNATIONAL JOURNAL OF THE JAPANESE OPHTHALMOLOGICAL SOCIETY

CLINICAL INVESTIGATION

Diquafosol sodium ophthalmic solution for the treatment of dry eye: clinical evaluation and biochemical analysis of tear composition

Chika Shigeyasu[1,2,3] · Masakazu Yamada[1] · Yoko Akune[3] · Kazuo Tsubota[2]

Received: 9 March 2015 / Accepted: 25 June 2015

Abstract
Purpose To evaluate the clinical efficacy of 3 % diquafosol sodium ophthalmic solution for dry eye, and to analyze the concentration of tear proteins and mucin-like substances after the treatment.
Methods Fifty eyes of 25 patients with dry eye syndrome were prospectively enrolled. The patients were treated with diquafosol solution at a dose of 1 drop in each eye 6 times daily for 4 weeks. The parameters of clinical efficacy were tear osmolarity, tear breakup time (BUT), fluorescein staining scores for the cornea and conjunctiva, Schirmer test values, and subjective symptoms evaluated using the ocular surface disease index (OSDI). Tears collected with Schirmer test strips were analyzed by high-performance liquid chromatography, and the concentrations of the total protein and the 4 major tear proteins, namely, secretory IgA, lactoferrin, lipocalin-1, lysozyme, and N-acetyl-neuraminic acid (Neu5Ac), were measured. Neu5Ac is a major sialic acid, a marker of secretory mucins.
Results The BUT, keratoconjunctival staining scores, and Schirmer test values were improved with statistical significance after the treatment with diquafosol solution, while changes in the other parameters, including tear osmolarity, corneal staining scores, and OSDI scores were not significant. The Neu5Ac concentration was significantly increased, which was not accompanied by changes in tear proteins.
Conclusions Topical application of diquafosol significantly improved the clinical parameters of the BUT, keratoconjunctival staining scores, and Schirmer test values and was accompanied by increased sialic acid content in the tears of patients with dry eye.

Keywords Diquafosol ophthalmic solution · Dry eye syndrome · Mucins · Tears · Sialic acid

Introduction

Recent investigations of dry eye have elucidated the cellular and molecular mechanisms of tear dysfunction and have led to the development of new therapies other than supplementation by ocular lubricants, with disease-related factors as the target [1]. One of these new therapies targeted to the biochemical mechanism is a $P2Y_2$ purinergic receptor agonist, diquafosol sodium. Diquafosol is more stable than other receptor agonists such as adenosine triphosphate and uridine 5′-triphosphate [2]. $P2Y_2$ receptors are present at various sites on the ocular surface, including goblet cells, the corneal epithelium, the meibomian glands, and ductal cells [3]. Binding of diquafosol to $P2Y_2$ receptors increases intracellular calcium concentrations and activates chloride ion transport, which drives fluid transport across the epithelial layer [4]. Studies have reported increased tear volume after the instillation of diquafosol in animal models [4, 5]. Increases in intracellular calcium concentrations induced by diquafosol also stimulate conjunctival goblet cell degranulation and the

✉ Chika Shigeyasu
shigeyasu@eye-center.org

1 Department of Ophthalmology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan

2 Department of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

3 Division for Vision Research, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8982, Japan

Published online: 27 August 2015

subsequent release of mucins to the ocular surface [6]. Fujihara et al. reported a decrease in periodic acid-Schiff-stained areas in rabbit conjunctival goblet cells obtained from impression cytology specimens after the instillation of diquafosol eye drops [7]. Nakamura et al. reported an increase in mucin secretion, which primarily consisted of MUC5AC, from the conjunctival tissues of rabbits as well as increased gene expression of membrane-binding mucin in cultured human corneal epithelial cells after diquafosol instillation [8].

Clinical studies have shown that topical instillation of diquafosol is effective for reducing corneal and conjunctival staining [8–11] and improving the tear film breakup time (BUT) [9, 10], and symptoms of dry eye [10]. Three percent diquafosol ophthalmic solution (Diquas ophthalmic solution 3 %; Santen Pharmaceutical, Osaka, Japan) was approved for clinical use by the Ministry of Health, Labour, and Welfare of Japan in December 2010. Since then, several studies have reported its clinical efficacy in specific subtypes of dry eye such as aqueous-deficient dry eye [12] and meibomian gland dysfunction [13]. Thus, diquafosol solution has become a major treatment modality for dry eye in Japan.

Diquafosol solution is believed to exert its therapeutic effect in dry eye patients by activating the $P2Y_2$ receptor in the conjunctiva, thereby promoting the secretion and content of aqueous and mucin in the tear fluids. Yokoi et al. recently reported that tear volume was increased after the instillation of diquafosol solution in normal human eyes [14]. We have also reported that diquafosol application increased the concentration of mucin-like substances in tears of healthy human research participants [15]. Hwang et al. recently reported an increase in the Schirmer value and goblet cell density after instillation of diquafosol in dry eye patients using the impression cytology method [16].

The aim of the current study was to prospectively determine the clinical efficacy of diquafosol solution for dry eye patients and to investigate the promotion of the secretion and content of mucin in the tear fluids by evaluating the concentration of tear proteins and mucin-like substances in the tear fluid after treatment.

Materials and methods

Study participants

All study participants received a diagnosis of dry eye according to the diagnostic criteria defined by the Japanese Dry Eye Society [17]. The diagnostic criteria of the Japanese Dry Eye Society are as follows: (1) one or more abnormal tear examination results (Schirmer I test $\leq$5 mm; BUT $\leq$5 s); (2) abnormal results on an ocular surface vital staining test (fluorescein corneal staining score $\geq$3); and (3) presence of dry eye symptoms. Participants who met 2 of the criteria (probable dry eye) and all 3 criteria (definite dry eye) were included in the study. The classification of aqueous tear deficiency dry eye and evaporative dry eye were made on the basis of the DEWS report [18]. The exclusion criteria were age <20 years, history of intraocular surgery within 90 days before the study, history of corneal surgery, and presence of active ocular surface disorders other than dry eye disease.

This study was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. The study participants received a full explanation of the procedures and provided their informed consent for participation before being included in the study. The protocol was approved by the institutional review board of the National Tokyo Medical Center (approval no. R10-022), and all study participants provided written informed consent.

Chemicals

Phosphate-buffered saline (PBS), bovine serum albumin, sodium phosphate dibasic, lactoferrin from human milk, and lysozyme from human neutrophils were obtained from Sigma-Aldrich (Tokyo, Japan). Secretory immunoglobulin A (sIgA) from human colostrum was obtained from Acris Antibodies (San Diego, CA, USA), and recombinant human lipocalin-1 was purchased from R&D Systems (Minneapolis, MN, USA). Advanced protein assay reagents were purchased from Cytoskeleton (Denver, CO, USA). Methanol, sodium chloride, and high-performance liquid chromatography (HPLC)-grade acetic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile (HPLC-grade) was obtained from Honeywell Japan (Tokyo, Japan), and 1,2-diamino-4,5-methylenedioxybenzene and coupling solutions (acetic acid, β-mercaptoethanol, and sodium hydrosulfite) were obtained from Takara Bio (Otsu, Japan).

Clinical assessment

At the baseline visit, 1 of the authors (CS) performed routine ocular examinations of all the study participants, followed by an examination of the ocular surface. Tear osmolarity was measured using the OcuSense TearLab Osmometer (TearLab; OcuSense, San Diego, CA, USA). Briefly, the tip of the pen was gently placed on the inferior temporal tear meniscus. After successful tear collection, the pen was replaced in the reader for measurement. Fluorescein solution (1 %; 1 μl) was used for measurement of the BUT. Corneal fluorescein staining with a 1 % fluorescein solution was assessed using the scoring system

described by Shimmura et al. in which the cornea is divided into 3 sections (superior, mid-cornea, and inferior) and graded on a scale of 0 (without any damage) to 3 (damage in the entire area) [19]. The total score ranged from 0 (minimum) to 9 (maximum) points. The fluorescein staining score of the keratoconjunctivitis sicca was determined using a blue-free barrier filter [20] according to the modified grading system of van Bijsterveld, in which each eye is divided into 3 sections (temporal conjunctiva, cornea, and nasal conjunctiva) and scored from 0 to 3 [21]. The final score ranged from 0 (minimum) to 9 (maximum) points. Tears were collected by inserting a Schirmer test strip (Alcon, Fort Worth, TX, USA) in the lower outer fornix without anesthesia. After 5 min, the strips were removed, the volume was measured, and each strip was immediately placed in a 1.5-ml Eppendorf tube and stored at −80 °C until the assay.

The symptoms of each participant were evaluated after objective observation using a validated Japanese translation of the ocular surface disease index (OSDI) questionnaire, which includes questions related to visual function, ocular symptoms, and environmental triggers. The scores were calculated according to the formula recommended by Schiffman et al. [22].

Diquafosol solution was prescribed. Patients were instructed to apply 1 drop to each eye 6 times a day for 4 weeks. Patients using artificial tears or sodium hyaluronate ophthalmic solution were instructed to continue use as needed. Some patients with Sjögren syndrome who were prescribed low-dose corticosteroid eye drops were instructed to continue with the same. The patients returned to the hospital after 4 weeks and underwent the same examinations performed at the baseline visit. The patients were instructed not to use diquafosol during the 1 h before the examination.

Tear protein assay

Before the assay, Schirmer strips were soaked in 200 µl of PBS for 30 min to elute the tear proteins. The procedures for analyzing the tear proteins were described in our previous reports [15, 23, 24]. In brief, total tear protein concentrations were determined by the Bradford method using a plate reader (Benchmark Plus; Bio-Rad Laboratories, Hercules, CA, USA) set at 590 nm. The 4 major tear proteins, namely, sIgA, lactoferrin, lipocalin-1, and lysozyme, were determined by fractioning each tear protein extract using an HPLC system consisting of an SCL-10Avp HPLC controller (Shimadzu Corporation, Kyoto, Japan), an LC-10ADvp solvent delivery system, an SPD-10Avp UV–VIS detector, and LC solution chromatography software (Shimadzu Corporation). Elution was performed with a TSK 3000SWXL column (7.8 mm i.d. × 300 mm; Tosoh Corporation, Tokyo, Japan), with a mobile phase of 0.5 M sodium chloride and 0.1 M sodium phosphate (pH 5.0). The flow rate was 0.7 ml/min, and the peaks were detected at 230 nm. The results were expressed as mg/ml.

Sialic acid assay

The concentration of N-acetyl-neuraminic acid (Neu5Ac) was measured. Neu5Ac is a major sialic acid in human bodies and has been used as a marker of mucins in human tear fluids. Procedures for analyzing sialic acids were based on the report of Yasueda et al. [25] and described in a previous report [15]. In brief, a volume of 10 µl of tear extract was mixed with 10 µl of 4 M acetic acid and kept at 80 °C for 3 h to release the sialic acid. A mixture of 200 µl of DMB solution, coupling solution, and water (ratio 1:5:4) was added, and the mixture was kept at 50 °C for 2.5 h in the dark to develop fluorescent labeling. The reaction mixture was cooled in ice water to stop the reaction and subjected to HPLC analysis.

Elution was performed using a COSMOSIL5C18-AR-2 column (4.6 mm i.d. × 150 mm; Nacalai Tesque, Kyoto, Japan) with a mobile phase of acetonitrile–methanol–water at a ratio of 2:14:84. The flow rate was 0.9 ml/min at 40 °C, and the peak of Neu5Ac was detected by fluorescence (excitation, 375 nm; emission, 448 nm). The Neu5Ac standard for quantification was obtained using a Sialic Acid Fluorescence Labeling kit (Takara Bio). The final results were expressed as µg/ml.

Statistical analysis

Data were analyzed using StatView version 5.0 software (SAS Institute, Cary, NC, USA), and the results were expressed as means and standard deviations (SD). The nonparametric Wilcoxon signed-rank test was used to compare pretreatment and posttreatment data. The Spearman rank correlation coefficient was used to identify associations among nonparametric variables and to evaluate correlations between sialic acid concentration and clinical measurements. The results were expressed as means ± SDs. Probability values lower than 0.05 were considered significant.

Results

Patient demographics

In total, 50 eyes of 25 patients were enrolled in the study. Several patients recognized an allowable range of slight irritation and increase in mucous discharge, and all successfully completed the trial and were included in the

statistical analysis. Sixteen patients had aqueous-deficient dry eye (12 patients with Sjögren syndrome and 4 patients with keratoconjunctivitis sicca), and 9 patients had evaporative dry eye. All the patients were female, with a mean age of 61.0 ± 16.2 years (range 24–82 years). The mean BUT, keratoconjunctival staining score, and Schirmer value were 1.7 ± 1.6 s, 2.2 ± 2.1 points, and 6.2 ± 4.4 mm, respectively. The baseline data of the clinical parameters are shown in Table 1.

Clinical efficacy

The changes in the clinical parameters after topical application of diquafosol 6 times a day for 4 weeks are shown in Table 1. Among these, the BUT ($P = 0.0001$), keratoconjunctival staining score ($P = 0.004$), and Schirmer value ($P = 0.022$) showed significant improvements (Wilcoxon signed-rank test). The tear osmolarity decreased from 292.6 ± 16.3 mOsm/l (range 27–349 mOsm/l) before treatment to 289.2 ± 10.7 mOsm/l (range 275–318 mOsm/l) after treatment, but without showing any significance. The changes in the other parameters including the corneal staining score and OSDI score were not significant.

Tear protein assay

The concentrations of the total protein and the 4 major tear proteins after diquafosol treatment for 4 weeks did not show any significant differences. The concentration of Neu5Ac, which represents sialic acid, increased significantly from 49.5 ± 40.3 to 76.7 ± 92.8 μg/ml ($P = 0.030$) after treatment (Table 2).

Among the clinical parameters, significant correlations were observed between the Neu5Ac concentration in tears and the BUT ($\rho = -0.565$, $P = 0.008$), corneal staining scores ($\rho = -0.666$, $P = 0.002$), and keratoconjunctival staining scores ($\rho = -0.617$, $P = 0.038$) before treatment (Spearman correlation coefficient).

Discussion

In the present study, we evaluated the clinical efficacy of diquafosol solution at a dose of 6 times daily for 4 weeks in patients with dry eye syndrome. The compositional changes in the tear proteins and mucin-like substances before and after the treatment were also analyzed. Among the clinical parameters examined, the BUT, keratoconjunctival staining scores, and Schirmer values showed significant improvements after diquafosol treatment for 4 weeks. Our results accord with those of previous reports [9–11]. In addition to these clinical efficacies, we have shown a significant increase in the sialic acid concentration in the tears of patients with dry eye, which has not been previously reported.

Mucins in the tears of patients with dry eye are reported to be decreased both quantitatively and qualitatively. Quantitative abnormalities include a decrease in sialic acid concentration in human tears [26] and a decrease in goblet cell density in the conjunctiva of patients with dry eye [27, 28]. With regard to qualitative abnormalities, a deficiency in the mucin granules of goblet cells and malformation of mucous carbohydrate chains has been reported in an animal model [29]. These deficiencies seem to result in the reduction of both secreted and membrane-associated mucins [30, 31].

In this study, we used sialic acid as a marker for ocular surface mucin. Sialic acid is often found in the nonreducing termini of mucous carbohydrate chains and has been used as a general marker of ocular surface mucin [25, 26]. Nakamura et al. reported the decrease in the sialic acid concentration of tears of dry eye patients [26] and Yasueda et al. reported the decrease in sialic acid in the tears of contact lens wearers [25]. The average concentration of

Table 1 Changes in the clinical parameters after topical application of 3 % diquafosol sodium ophthalmic solution 6 times daily for 4 weeks

Clinical measures	Pretreatment (50 eyes, 25 patients)	Posttreatment (50 eyes, 25 patients)	*P* value
Tear osmolarity, mOsm/l	292.6 ± 16.3	289.2 ± 10.7	0.256
Tear breakup time, s	1.7 ± 1.6	3.1 ± 1.7	0.0001***
Corneal staining score/9	1.7 ± 1.5	1.4 ± 1.8	0.364
Keratoconjunctival staining score/9	2.2 ± 2.1	1.3 ± 1.7	0.004**
Schirmer value, mm	6.2 ± 4.4	7.5 ± 5.0	0.022*
OSDI scoring[a]/100	38.7 ± 17.8	33.9 ± 22.5	0.134

Data are presented as means ± standard deviations

The values of each parameter before and after application were compared

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Wilcoxon signed-rank test)

[a] Data in number of patients

Table 2 Changes in tear protein content after topical application of 3 % diquafosol sodium ophthalmic solution 6 times daily for 4 weeks

Tear proteins	Pretreatment	Posttreatment	*P* value
Total protein, mg/ml	8.20 ± 5.42	8.01 ± 5.72	0.932
sIgA, mg/ml	0.56 ± 0.46	0.64 ± 0.51	0.153
Lactoferrin, mg/ml	3.53 ± 2.47	2.89 ± 1.65	0.153
Lipocalin, mg/ml	1.37 ± 0.69	1.78 ± 1.83	0.189
Lysozyme, mg/ml	0.77 ± 0.43	1.05 ± 1.14	0.241
Neu5Ac, μg/ml	49.5 ± 40.3	76.7 ± 92.8	0.030*

Data are presented as means ± standard deviations

The contents of each tear protein before and after treatment were compared

* $P < 0.05$ (Wilcoxon signed-rank test)

sialic acid in the tears of dry eye patients before the treatment in this study was 49.5 ± 40.3 μg/ml, which was lower than that in our previous report among healthy study participants [15] and in agreement with the report of Nakamura et al. [26]. Furthermore, the sialic acid concentration was significantly correlated with the BUT, corneal staining score, and keratoconjunctival staining score. This result seems to support the notion that decrease in mucin may play a role in the pathophysiology of dry eye, which suggests that the concentration of sialic acid in tears may be a good marker of the condition and severity of dry eye.

The most important and novel finding in the current study was the observation that the sialic acid concentration in the tears of patients with dry eye was increased after diquafosol application for 4 weeks. Because we have previously reported the immediate response from significant increase of sialic acid concentration in tears of healthy study participants after a single application of diquafosol solution [15], it was very interesting to find out the chronic effect on clinical efficacy among dry eye patients. The chronic effect may be exerted through the improvement of dry eye conditions by diquafosol, which results in the increase and improvement of conjunctival goblet cells. We could not specify the type of mucins present because we adopted sialic acid as a general marker of mucins. However, according to previous reports, secreted mucins are likely the major source of sialic acid in tears induced by diquafosol [6, 8]. Secreted mucins from the conjunctival goblet cells are thought to form a thick and loose mucous blanket that covers the ocular surface epithelia [32], which plays a crucial role in maintaining tear film stability. The significant improvement in the BUT of patients with dry eye shown by this study and by previous studies may support this hypothesis.

The possible limitation of the current study was its study design, namely, a prospective cohort study. There was no control arm for comparison. Patients were instructed to keeping using the same regimen as they had been using, including artificial tears, sodium hyaluronate ophthalmic solution, and corticosteroid eye drops. In addition, our current study had a rather small number of study participants. We included both patients with aqueous-deficient dry eye and evaporative dry eye. Although diquafosol appears to be effective for both types of dry eye according to its pharmacologic actions and reported clinical efficacy, further studies to define the issue should be considered. It would also have been interesting to study a group of severe dry eye patients. Diquafosol treatment in patients with severe ocular surface damage may have produced different results. Although our study included 12 patients with Sjögren syndrome, their dry eye was relatively mild in terms of clinical severity. The number of participants in our study was insufficient for subgroup analysis. Further studies are required to clarify these issues.

In conclusion, we were able to evaluate the improvement in the clinical parameters and the sialic acid concentration in the tears of dry eye patients after diquafosol instillation. Further studies should evaluate diquafosol-induced increases in tear and mucin secretion in the different types of dry eye. Suitable indications for diquafosol administration in patients with dry eye should also be further investigated.

Conflicts of interest C. Shigeyasu, None; M. Yamada, None; Y. Akune, None; K. Tsubota, Lecture fees (Santen Pharmaceutical Co., Ltd.).

References

1. Pflugfelder SC, Geerling G, Kinoshita S, Lemo MA, McCulley J, Nelson D, et al. Management and therapy of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye Workshop (2007). Ocul Surf. 2007;5:163–78.
2. Nichols KK, Yerxa B, Kellerman DJ. Diquafosol tetrasodium: a novel dry eye therapy. Expert Opin Investig Drugs. 2004;13:47–54.
3. Cowlen MS, Zhang VZ, Warnock L, Moyer CF, Peterson WM, Yerxa BR. Localization of ocular P2Y2 receptor gene expression by in situ hybridization. Exp Eye Res. 2003;77:77–84.
4. Murakami T, Fujihara T, Horibe Y, Nakamura M. Diquafosol elicits increases in net Cl-transport through P2Y2 receptor stimulation in rabbit conjunctiva. Ophthalmic Res. 2004;36:89–93.
5. Takaoka-Shichijyou Y, Murakami T, Nakamura M. Stimulatory effect of diquafosol tetrasodium on tear fluid secretion in normal rabbits [in Japanese]. Atarashii Ganka. 2011;28:1029–33.
6. Jumblatt JE, Jumblatt MM. Regulation of ocular mucin secretion by P2Y2 nucleotide receptors in rabbit and human conjunctiva. Exp Eye Res. 1998;67:341–6.
7. Fujihara T, Murakami T, Nagano T, Nakamura M, Nakata K. INS365 suppresses loss of corneal epithelial integrity by secretion of mucin-like glycoprotein in a rabbit short-term dry eye model. J Ocul Pharmacol Ther. 2002;18:363–70.

8. Nakamura M, Imanaka T, Sakamoto A. Diquafosol ophthalmic solution for dry eye treatment. Adv Ther. 2012;29:579–89.
9. Takamura E, Tsubota K, Watanabe H, Ohashi Y. A randomised, double-masked comparison study of diquafosol versus sodium hyaluronate ophthalmic solutions in dry eye patients. Br J Ophthalmol. 2012;96:1310–5.
10. Matsumoto Y, Ohashi Y, Watanabe H, Tsubota K. Efficacy and safety of diquafosol ophthalmic solution in patients with dry eye syndrome: a Japanese phase 2 clinical trial. Ophthalmology. 2012;119:1954–60.
11. Tauber J, Davitt WF, Bokosky JE, Nichols KK, Yerxa BR, Schaberg AE, et al. Double-masked, placebo-controlled safety and efficacy trial of diquafosol tetrasodium (INS365) ophthalmic solution for the treatment of dry eye. Cornea. 2004;23:784–92.
12. Koh S, Maeda N, Ikeda C, Oie Y, Soma T, Tsujikawa M, et al. Effect of diquafosol ophthalmic solution on the optical quality of the eyes in patients with aqueous-deficient dry eye. Acta Ophthalmol. 2014;92:e671–5.
13. Arita R, Suehiro J, Haraguchi T, Maeda S, Maeda K, Tokoro H, et al. Topical diquafosol for patients with obstructive meibomian gland dysfunction. Br J Ophthalmol. 2013;97:725–9.
14. Yokoi N, Kato H, Kinoshita S. Facilitation of tear fluid secretion by 3% diquafosol ophthalmic solution in normal human eyes. Am J Ophthalmol. 2014;157:85–92.
15. Shigeyasu C, Hirano S, Akune Y, Yamada M. Diquafosol tetrasodium increases the concentration of mucin-like substances in tears of healthy human subjects. Curr Eye Res. 2014;. doi:10.3109/02713683.2014.967871.
16. Hwang HS, Sung YM, Lee WS, Kim EC. Additive effect of preservative-free sodium hyaluronate 0.1% in treatment of dry eye syndrome with diquafosol 3% eye drops. Cornea. 2014;33:935–41.
17. Shimazaki J, Tsubota K, Kinoshita S, Ohashi Y, Shimomura Y, Tagawa Y, et al. Definition and diagnosis of dry eye 2006 [in Japanese]. Atarashii Ganka. 2007;24:181–4.
18. Lemp MA, Baudouin C, Baum J, Dogru M, Foulks GN, Kinoshita S, et al. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye Workshop (2007). Ocul Surf. 2007;5:75–92.
19. Shimmura S, Ono M, Shinozaki K, Toda I, Takamura E, Mashima Y, et al. Sodium hyaluronate eyedrops in the treatment of dry eyes. Br J Ophthalmol. 1995;79:1007–11.
20. Koh S, Watanabe H, Hosohata J, Hori Y, Hibino S, Nishida K, et al. Diagnosing dry eye using a blue-free barrier filter. Am J Ophthalmol. 2003;136:513–9.
21. van Bijsterveld OP. Diagnostic tests in the sicca syndrome. Arch Ophthalmol. 1969;82:10–4.
22. Schiffman RM, Christianson MD, Jacobsen G, Hirsch JD, Reis BL. Reliability and validity of the ocular surface disease index. Arch Ophthalmol. 2000;118:615–21.
23. Yamada M, Mochizuki H, Kawai M, Tsubota K, Bryce TJ. Decreased tear lipocalin concentration in patients with meibomian gland dysfunction. Br J Ophthalmol. 2005;89:803–5.
24. Shigeyasu C, Hirano S, Akune Y, Mochizuki H, Yamada M. Evaluation of the frequency of ophthalmic solution application: washout effects of topical saline application on tear components. Curr Eye Res. 2013;38:722–8.
25. Yasueda S, Yamakawa K, Nakanishi Y, Kinoshita M, Kakehi K. Decreased mucin concentrations in tear fluids of contact lens wearers. J Pharm Biomed Anal. 2005;39:187–95.
26. Nakamura Y, Yokoi N, Tokushige H, Kinoshita S. Sialic acid in human tear fluid decreases in dry eye. Jpn J Ophthalmol. 2004;48:519–23.
27. Kinoshita S, Kiorpes TC, Friend J, Thoft RA. Goblet cell density in ocular surface disease: a better indicator than tear mucin. Arch Ophthalmol. 1983;101:1284–7.
28. Ralph RA. Conjunctival goblet cell density in normal subjects and in dry eye syndromes. Invest Ophthalmol. 1975;14:299–302.
29. Corrales RM, de Paiva CS, Li DQ, Farley WJ, Henriksson JT, Bergmanson JP, et al. Entrapment of conjunctival goblet cells by desiccation-induced cornification. Invest Ophthalmol Vis Sci. 2011;52:3492–9.
30. Argueso P, Balaram M, Spurr-Michaud S, Keutmann HT, Dana MR, Gipson IK. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. Invest Ophthalmol Vis Sci. 2002;43:1004–11.
31. Danjo Y, Watanabe H, Tisdale AS, George M, Tsumura T, Abelson MB, et al. Alteration of mucin in human conjunctival epithelia in dry eye. Invest Ophthalmol Vis Sci. 1998;39:2602–9.
32. Gipson IK, Hori Y, Argueso P. Character of ocular surface mucins and their alteration in dry eye disease. Ocul Surf. 2004;2:131–48.

OPEN

Citation: Human Genome Variation (2015) 2, 15047; doi:10.1038/hgv.2015.47
Official journal of the Japan Society of Human Genetics 2054-345X/15

www.nature.com/hgv

DATA REPORT

Novel *TACSTD2* mutation in gelatinous drop-like corneal dystrophy

Passara Jongkhajornpong[1], Kaevalin Lekhanont[1], Mayumi Ueta[2,3], Koji Kitazawa[2], Satoshi Kawasaki[4] and Shigeru Kinoshita[3]

We identified a novel mutation in the tumor-associated calcium signal transducer 2 (*TACSTD2*) gene in a consanguineous Thai family with gelatinous drop-like corneal dystrophy (GDLD). All affected family members presented with an intense amyloid substance deposited on the cornea, which required surgical management. Genetic analysis of these individuals revealed a homozygous mutation c.79delC, in the *TACSTD2* gene. Both parents of these individuals were unaffected and showed heterozygous mutations in the *TACSTD2* gene. The mutation produced a truncated protein sequence that might be the cause of GDLD.

Human Genome Variation (2015) **2,** 15047; doi:10.1038/hgv.2015.47; published online 26 November 2015

GDLD is a rare autosomal recessive hereditary disease. It was first reported in 1914, by Nakaizumi[1–3] and most of the subsequent reports of GDLD have originated from Japan. People with GDLD exhibit a wide variety of clinical presentations, and GDLD has been classified into four types: (1) typical mulberry type, (2) kumquat-like type, (3) band-keratopathy type and (4) stromal-opacity type.[4] The onset of GDLD occurs in the first or second decade of life, and patients usually require surgical treatment in the third decade of life. In 1999, Tsujikawa *et al.*[5] were the first to identify *TACSTD2* as the pathologic gene of GDLD. Various mutations have been discovered in the *TACSTD2* gene in various populations.[6–15] To date, there are 28 reported mutations in the *TACSTD2* gene. For Southeast Asian populations, one *TACSTD2* mutation has been reported in Vietnam[11] and, to the best of our knowledge, we are the first to investigate the genetic mutation of the *TACSTD2* gene in Thailand.

Three GDLD probands (two males and one female) from the same consanguineous family (pedigree shown in Figure 1) were recruited for this study. The family was from Chiang Mai, which is a province in northern Thailand. There was no associated systemic disease or history of corneal trauma in the family, and the ocular examination in the parents and all offspring of the probands appeared normal. The clinical history, presentation and pathologic data for each proband were as follows:

Proband 1 (II-1) is a 39-year-old male with bilateral corneal opacity and horizontal nystagmus. His best spectacle-corrected visual acuity was counting fingers at a distance of 1 foot with both eyes. The onset of his symptoms occurred in the first decade of life. He had previously undergone three times of penetrating keratoplasties (PKP) on the right eye and twice on the left eye at another hospital. Ocular examination revealed multiple gray-to-yellowish nodules deposited in the corneal stroma in both the graft and host cornea (Figure 2a, b). We performed another PKP on his right eye and type 1 Boston keratoprosthesis (KPro) implantation (Massachusetts Eye and Ear Infirmary, Boston, MA, USA) on his left eye. Graft rejection was detected 4 months after the PKP operation. Graft failure subsequently occurred. The visual outcome of Boston KPro implantation was limited to counting fingers at a

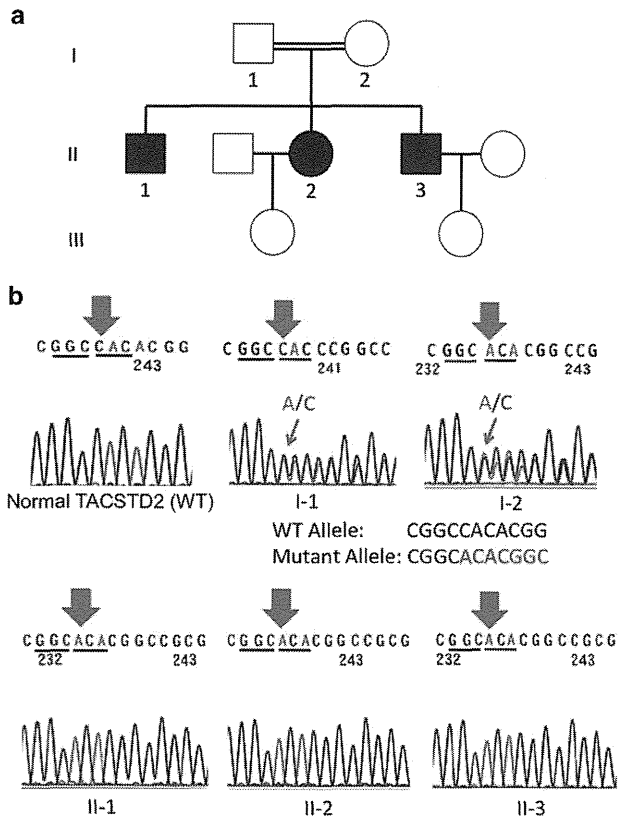

Figure 1. Family tree of a gelatinous drop-like corneal dystrophy (GDLD) consanguineous Thai family (**a**). Diagrams illustrating the nucleotide sequences of the tumor-associated calcium signal transducer 2 (*TACSTD2*) genes of all family members compared with the normal *TACSTD2* gene (wild type, WT) (**b**). Arrows indicate the seventy-ninth nucleotide of the *TACSTD2* gene, which exhibited a heterozygous deletion in both parents and a homozygous deletion in all three probands.

[1]Department of Ophthalmology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand; [2]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; [3]Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan and [4]Department of Ophthalmology, Osaka University, Osaka, Japan.
Correspondence: P Jongkhajornpong (passaraj@yahoo.com)
Received 2 June 2015; revised 30 September 2015; accepted 1 October 2015

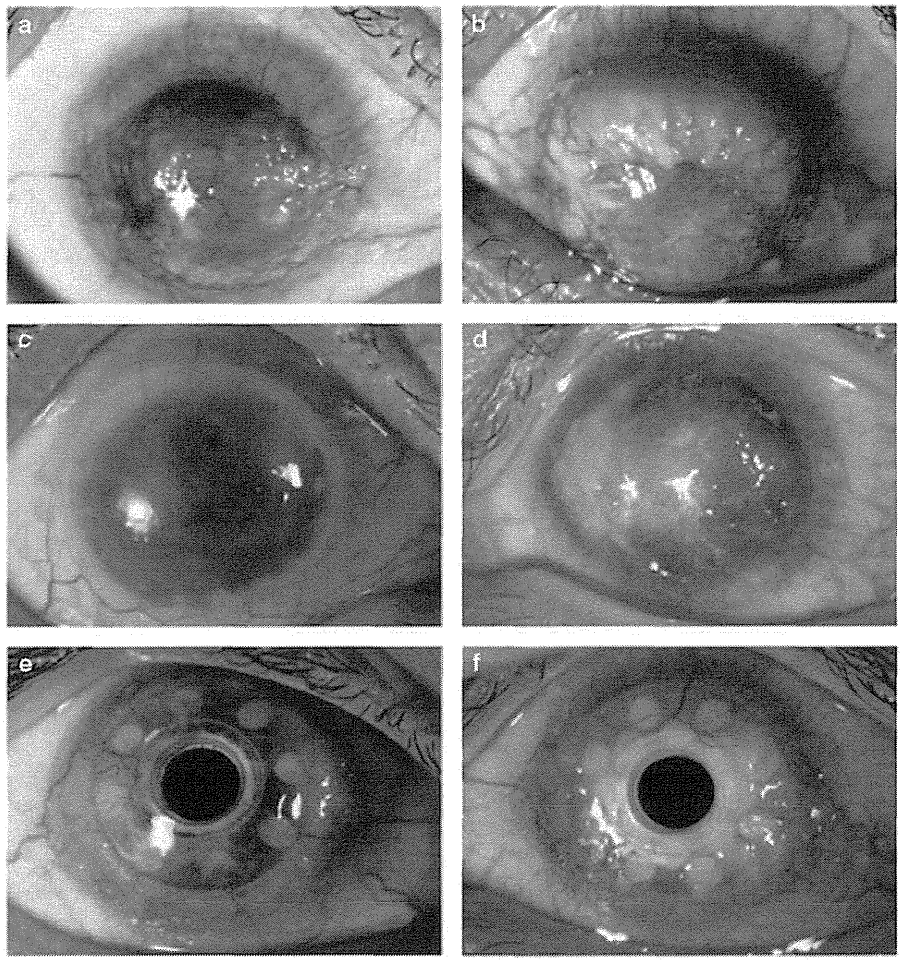

Figure 2. Slit-lamp examination of the three gelatinous drop-like corneal dystrophy (GDLD) patients. Proband 1: recurrent mulberry type in the right eye (**a**) and kumquat-like type in the left eye (**b**), Proband 2: post penetrating keratoplasty (PKP) in the right eye (**c**) and recurrent mulberry-type amyloid deposit in the left eye (**d**), Proband 3: post Boston keratoprosthesis (KPro) implantation in the right eye (**e**) and the left eye (**f**).

distance of 2 feet with his left eye owing to severe underlying amblyopia.

Proband 2 (II-2) is a 33-year-old female with a recurrent amyloid deposit who had previously undergone a PKP of her right eye once and of her left eye twice at another hospital. A mulberry-type amyloid deposit was found during the ocular examination (Figure 2d). We performed type 1 Boston KPro implantation on her right eye. Six years post operation, a severe *Pseudomonas* infection occurred in her right eye. The Boston KPro was removed and replaced with a corneal graft, which ultimately failed after 1 year (Figure 2b).

Proband 3 (II-3) is a 28-year-old male with a bilateral mulberry-type amyloid deposit. For treatment, PKP was performed on his right eye. Recurrence of the yellowish amyloid deposit subsequently occurred. Therefore, we performed a type 1 Boston KPro implantation as the secondary treatment on his right eye and as the primary treatment on his left eye (Figure 2e, f).

Histopathology of the corneal tissue showed drops and bands of amorphous material that were deposited at the subepithelial and anterior corneal stroma (Figure 3a, c for proband 1 and proband 3, respectively) and were positive for Congo red staining with birefringence consistent with amyloid (Figure 3b, d for proband 1 and proband 3, respectively).

Mutational analyses were performed on all of the family members (the three probands and both parents) after obtaining informed consent. Ten subjects without corneal amyloid deposit were recruited as a control group. Genomic DNA was extracted from 8 ml of whole peripheral blood using the PAXgeneBlood DNA Kit (QIAGEN GmbH, Hilden, Germany). DNA templates were quantified to be 150–300 ng before use in the reaction. PCR was performed using the following primer pair to cover the entire*TACSTD2* gene: F; 5′-ATGTGTCACCCAAATACCAGTGGG-3′ and R; 5′-CGTGACTCACTTGGGTCTGGGACG-3′. After purification of the PCR products, the products were bi-directionally sequenced using the primers with a cycle sequencing kit (BigDye Terminator Cycle Sequencing Kit; Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After ethanol precipitation, the products were electrophoresed using an automated capillary sequencer (3130xl Genetic Analyzer; Life Technologies). The sequencing data were validated by PCR using a primer pair (intF: 5′-AGTGCAACCAGACGTCGGTGTGCT-3′ and intR: 5′-TCTGAGACGTGTTCTGCCGCAGCT-3′) that spanned the site of the identified mutation.

By using direct DNA sequencing, homozygous c.79delC mutation (p.His27ThrfsTer15) in the *TACSTD2* gene was found in all three patients (Figure 1). Heterozygous c.79delC mutation was confirmed in both of the parents who were unaffected.

In this report, we discovered a novel homozygous c.79delC mutation in the *TACSTD2* gene of three Thai GDLD patients that is not present in the wild-type *TACSTD2* gene (Figure 1) of Thai controls or in the Exome Aggregation Consortium database, which includes data from 60,706 unrelated individuals.[16] The autosomal

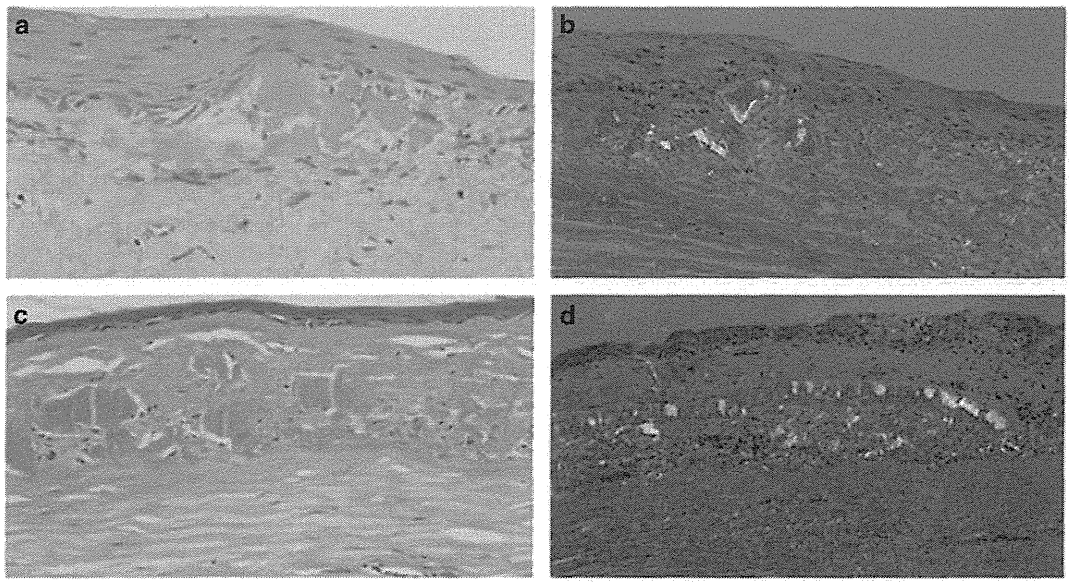

Figure 3. Histopathology images of the corneal tissues. Hematoxylin and eosin staining revealed multiple amyloid nodules deposited in the subepithelium and the anterior stroma in both proband 1 (**a**) and proband 3 (**c**). Using Congo red staining, an apple-green birefringence was observed under polarized light in both proband 1 (**b**) and proband 3 (**d**).

recessive hereditary pattern of GDLD was confirmed by detection of heterozygous c.79delC mutation of the *TACSTD2* gene in both parents who were unaffected. This novel deletion (c.79delC) caused a frameshift mutation that resulted in an early stop codon. The resultant TACSTD2 molecule contained a threonine instead of a histidine at the 27th amino acid, with the new open reading frame encoding 14 amino acids (p.His27 Thrfs Ter15) before termination. The aberrant protein contained 41 amino acids. Generally, the TACSTD2 molecule is composed of five main regions: (1) a signal sequence (located at amino acid (aa) 1–26), (2) an epidermal growth factor-like repeat, (3) a thyroglobulin repeat, (4) a transmembrane domain (TM, located at aa 275–297) and (5) a phosphatidylinositol 4,5-bis phosphate (PIP2)-binding sequence.[5] It has been proposed that the transmembrane domain plays a role in anchoring the molecule to tight junction-related proteins via the AxxxG motif.[17] Furthermore, knocking out *TACSTD2* in human corneal and conjunctival epithelial cells has been shown to result in a significant decrease in transepithelial resistance and a low expression of Claudin 1 and 7.[18,19] Investigation of the truncated TACSTD2 molecule identified in this study revealed that four of the necessary functional domains of the molecule had disappeared, including the TM domain. We posited that the aberrant TACSTD2 molecule was unable to bind to the plasma membrane or function properly, thus resulting in the clinical appearance of GDLD.

The most frequently reported mutation in the *TACSTD2* gene in Japan is p.Gln118X, which accounts for 82.5% of all mutations in the *TACSTD2* gene.[5] However, p.Glu227Lys is reported to be the most common mutation among Iranian GDLD patients.[12] This phenomenon can be explained through a founder effect, as shown by analysis of polymorphic markers near the *TACSTD2* gene. A few transethnic mutations have been discovered, such as p.Gln118X in Japanese and Chinese populations, p.Leu186Pro in Japanese and Iranian populations and p.Ile258X 772_783del +772insT in Vietnamese and Chinese populations. Uncovering a unique *TACSTD2* mutation in people from these nations, including Thai GDLD patients is strong evidence of high genotypic heterogeneity of GDLD. As no other nucleotide variations were recognized in the *TACSTD2* region, we did not find a founder effect in Thai GDLD patients.

Regarding the relationship between genotypes and phenotypes, all patients in the present study carried the same mutation; however, they presented with different ocular findings, both inter-individually and intra-individually. Proband 1 presented with a mulberry-type amyloid deposit in his right eye and a kumquat-like type in his left eye (Figure 2). The onset of the disease in proband 1 was much earlier than that of his brother and sister, who initially experienced the symptoms in their second decade of life. Similar to the previous study of Tsujikawa *et al.*,[20] we observed high phenotypic variability in a single family with GDLD.

In conclusion, we investigated the clinical features, histopathology and genetics of three GDLD patients from Thailand and discovered a unique mutation, c.79delC, in the *TACSTD2* gene. This frameshift mutation produced a truncated TACSTD2 protein that might cause the severe clinical presentation, which was phenotypically heterogeneous and required surgical management.

HGV DATABASE

The relevant data from this Data Report are hosted at the Human Genome Variation Database at http://dx.doi.org/10.6084/m9.figshare.hgv.741.

ACKNOWLEDGEMENTS

We thank Dr Rangsima Aroonroch, Dr Varintorn Chuckpaiwong, Dr Pichaya Chuepanich, Dr Nutthida Nimvorapun and Dr Manachai Manasopon from Ramathibodi Hospital, Mahidol University, Bangkok, Thailand, for the tissue pathological reports and clinical data; Dr Morio Ueno from the Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan, for assisting in the DNA sequencing; the Exome Aggregation Consortium; and the groups that provided the exome variant data for comparison. A full list of contributing groups can be found at http://exac.broadinstitute.org/about. Ethics approval was obtained from the Institutional Review Board for Human Studies of Ramathibodi Hospital, Mahidol University, Bangkok, Thailand, and the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine, Kyoto, Japan. This research project was supported by the Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

COMPETING INTERESTS

The authors declare no conflict of interest.

REFERENCES

1 Weiss JS, Møller HU, Aldave AJ, Seitz B, Bredrup C, Kivela T *et al.* IC3D Classification of corneal dystrophies-edition 2. *Cornea* 2015; **34**: 117–159.
2 Nakaizumi G. A rare case of corneal dystrophy. *Acta Soc Ophthalmol Jpn* 1914; **18**: 949–950.
3 Kawano H, Fujiki K, Kanai A. Prevalence of gelatinous drop-like corneal dystrophy in Japan [in Japanese]. *Atarashii Ganka* 1992; **9**: 1879–1882.
4 Ide T, Nishida K, Maeda N, Tsujikawa M, Yamamoto S, Watanabe H *et al.* A spectrum of clinical manifestations of gelatinous drop-like corneal dystrophy in Japan. *Am J Ophthalmol* 2004; **137**: 1081–1084.
5 Tsujikawa M, Kurahashi H, Tanaka T, Nishida K, Shimomura Y, Tano Y *et al.* Identification of the gene responsible for gelatinous drop-like corneal dystrophy. *Nat Genet* 1999; **21**: 420–423.
6 Nakatsukasa M, Kawasaki S, Yamasaki K, Fukuoka H, Matsuda A, Nishida K *et al.* Two novel mutations of TACSTD2 found in three Japanese gelatinous drop-like corneal dystrophy families with their aberrant subcellular localization. *Mol Vis* 2011; **17**: 965–970.
7 Kinoshita S, Adachi W, Sotozono C, Nishida K, Yokoi N, Quantock AJ *et al.* Characteristics of the human ocular surface epithelium. *Prog Retin Eye Res* 2001; **20**: 639–673.
8 Ren Z, Lin PY, Klintworth GK, Iwata F, Munier FL, Schorderet D. Allelic and locus heterogeneity in autosomal recessive gelatinous drop-like corneal dystrophy. *Hum Genet* 2002; **110**: 568–577.
9 Markoff A, Bogdanova N, Uhlig CE, Groppe M, Horst J, Kennerknecht I. A novel TACSTD2 gene mutation in a Turkish family with a gelatinous drop-like corneal dystrophy. *Mol Vis* 2006; **12**: 1473–1476.
10 Tasa G, Kals J, Muru K, Juronen E, Piirsoo A, Veromann S *et al.* A novel mutation in the M1S1 gene responsible for gelatinous droplike corneal dystrophy. *Invest Ophthalmol Vis Sci* 2001; **42**: 2762–2764.
11 Ha NT, Chau HM, Cung le X, Thanh TK, Marukami A, Kanai A. A novel mutation of M1S1 gene found in a Vietnamese patient with gelatinous droplike corneal dystrophy. *Am J Ophthalmol* 2003; **135**: 390–393.
12 Alavi A, Elahi E, Tehrani MH, Amoli FA, Javadi MA, Rafati N *et al.* Four mutations (three novel, one founder) in TACSTD2 among Iranian GDLD patients. *Invest Ophthalmol Vis Sci* 2007; **48**: 4490–4497.
13 Jing Y, Liu C, Wang L. A novel TACSTD2 mutation identified in two Chinese brothers with gelatinous drop-like corneal dystrophy. *Mol Vis* 2009; **15**: 1580–1588.
14 Zhang B, Yao YF. Gelatinous drop-like corneal dystrophy with a novel mutation of TACSTD2 manifested in combination with spheroidal degeneration in a Chinese patient. *Mol Vis* 2010; **16**: 1570–1575.
15 Paliwal P, Gupta J, Tandon R, Sharma N, Titiyal JS, Kashyap S *et al.* Identification and characterization of a novel TACSTD2 mutation in gelatinous drop-like corneal dystrophy. *Mol Vis* 2010; **16**: 729–739.
16 Exome Aggregation Consortium (ExAC), Cambridge, MA, USA http://exac.broadinstitute.org. Accessed 29 September 2015; Updated on 29 October 2014.
17 Nakatsukasa M, Kawasaki S, Yamasaki K, Fukuoka H, Matsuda A, Tsujikawa M *et al.* Tumor-associated calcium signal transducer 2 is required for the proper subcellular localization of claudin 1 and 7: implications in the pathogenesis of gelatinous drop-like corneal dystrophy. *Am J Pathol* 2010; **177**: 1344–1355.
18 Kinoshita S, Kawasaki S, Kitazawa K, Shinomiya K. Establishment of a human conjunctival epithelial cell line lacking the functional TACSTD2 gene (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc* 2012; **110**: 166–177.
19 Kitazawa K, Kawasaki S, Shinomiya K, Aoi K, Matsuda A, Funaki T *et al.* Establishment of a human corneal epithelial cell line lacking the functional TACSTD2 gene as an in vitro model for gelatinous drop-like dystrophy. *Invest Ophthalmol Vis Sci* 2013; **54**: 5701–5711.
20 Tsujikawa M, Maeda N, Tsujikawa K, Hori Y, Inoue T, Nishida K. Chromosomal sharing in atypical cases of gelatinous drop-like corneal dystrophy. *Jpn J Ophthalmol* 2010; **54**: 494–498.