**Objective:** To report phenotypic analysis of epithelia before and following cultivated limbal epithelial transplantation (CLET) for eyes with limbal stem cell deficiency.

**Methods:** Six patients with limbal stem cell deficiency (3 with alkali burns, 2 with Stevens-Johnson syndrome, and 1 with pseudo-ocular cicatricial pemphigoid) were subjected to CLET and subsequent keratoplasty. Immunohistochemical analysis for cytokeratin 3, cytokeratin 13, MUC5AC (Mucin 5 subtype AC), and /H9251 smooth muscle actin was performed in specimens obtained at CLET and keratoplasty. Clinical outcome was assessed according to epithelial phenotype, visual acuity, neovascularization, and graft clarity.

**Results:** Secondary keratoplasty was performed following CLET during a mean interval of 6.8 months. Postoperative visual acuity improved by more than 2 lines over a mean±SD observation period of 25.1±13.2 months following keratoplasty, with reduction of neovascularization. Phenotypic study revealed that epithelia were positive for cytokeratin 13 and MUC5AC, but negative for cytokeratin 3, with a smooth muscle actin–positive cells in the stroma in all patients before CLET. After CLET, 4 eyes showed positive immunostaining to cytokeratin 3 but negative immunostaining to cytokeratin 13 and MUC5AC, with no α smooth muscle actin–positive cells.

**Conclusion:** Cultivated limbal epithelial transplantation is a useful approach in the treatment of limbal stem cell deficiency, restoring a feasible microenvironment in the ocular surface and securing a corneal epithelial phenotype.

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**Clinically**, limbal stem cell deficiency (LSCD), which can arise from problems such as Stevens-Johnson syndrome or chemical burns, is one of the most challenging of disorders. In many cases, such diseases completely destroy limbal epithelial stem cells, their niche, or a combination of both. Corneas with limbal deficiency demonstrate conjunctivalization, vascularization, and chronic inflammation.\(^1\)\(^-\)\(^4\)

In such cases, when penetrating keratoplasty (PKP) is performed without stabilization of chronic inflammation or improvement of the microenvironment of the corneal epithelia, surgery yields a high rate of failure.\(^5\)\(^-\)\(^6\) A combination of limbal and amniotic membrane (AM) transplantation stabilizes ocular surface inflammation and provides a supply of limbal progenitor cells. Although this approach has substantially improved the surgical prognosis of patients with LSCD,\(^7\)\(^-\)\(^11\) the long-term success rate has remained at approximately 50%, according to recent studies.\(^7\)\(^,\)\(^9\)\(^,\)\(^10\) Common causes of failure have included insufficient regeneration of corneal epithelial cells and immunological rejection.

Recent advances in regenerative medicine have allowed us to transplant limbal epithelial sheets ex vivo onto denuded AM, which only requires a small piece of donor tissue. The advantage of this method over conventional limbal transplantation is that it allows potential complications in the donor eye to be avoided.\(^12\) The use of various culture techniques and/or carriers has yielded encouraging results. However, there have been few studies on the relationship between clinical outcome and epithelial phenotype. Recently, Sangwan et al\(^13\) reported that a stable ocular surface was achieved in 57 of 78 eyes (73%) subjected to autologous cultivated limbal epithelial transplantation (auto-CLET), which was confirmed by immunohistochemical analysis for cytokeratin 3 (K3).\(^14\) However, they did not demonstrate epithelial phenotype before CLET, so it is not clear whether the regeneration of the corneal phenotype epithelium was attributable to CLET. Herein, we studied the phenotype of the corneal epithe-
Table 1. Patient Demographic Profiles

<table>
<thead>
<tr>
<th>Patient No./ Sex/Age, y</th>
<th>Original Disease or Problem</th>
<th>Pre-CLET BCVA</th>
<th>Conjunctivalization, %</th>
<th>Schirmer Value, mm</th>
<th>Other Diseases</th>
<th>Source of Donor Tissue for CLET</th>
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</thead>
<tbody>
<tr>
<td>1/M/24</td>
<td>Alkali Burns</td>
<td>20/666</td>
<td>80</td>
<td>25</td>
<td>NA</td>
<td>Ipsilateral autologous</td>
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<tr>
<td>2/M/44</td>
<td>Alkali Burns</td>
<td>HM</td>
<td>100</td>
<td>5</td>
<td>Entropion</td>
<td>Nonrelated allogeneic</td>
</tr>
<tr>
<td>3/M/64</td>
<td>Alkali Burns</td>
<td>20/1000</td>
<td>100</td>
<td>21</td>
<td>NA</td>
<td>Nonrelated allogeneic</td>
</tr>
<tr>
<td>4/F/76</td>
<td>SJS</td>
<td>HM</td>
<td>100</td>
<td>2</td>
<td>Severe MGD symblepharon</td>
<td>Nonrelated allogeneic</td>
</tr>
<tr>
<td>5/F/82</td>
<td>SJS</td>
<td>CF</td>
<td>100</td>
<td>7</td>
<td>Symblepharon</td>
<td>Living relative allogeneic</td>
</tr>
<tr>
<td>6/M/54</td>
<td>Pseudo-OPC</td>
<td>HM</td>
<td>100</td>
<td>25</td>
<td>Symblepharon</td>
<td>Contralateral autologous</td>
</tr>
</tbody>
</table>

Abbreviations: BCVA, best-corrected visual acuity; CF, counting fingers; CLET, cultivated limbal epithelial transplantation; HM, hand motions; MGD, meibomian gland dysfunction; NA, not applicable; OCP, ocular cicatricial pemphigoid; SJS, Stevens-Johnson syndrome.

Table 2. Surgical Data

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Post-CLET BCVA</th>
<th>Duration Between CLET and KP, mo</th>
<th>Type of KP</th>
<th>Additional Surgery</th>
<th>Postkeratoplasty Data</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BCVA</td>
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<tr>
<td>1</td>
<td>20/500</td>
<td>2</td>
<td>PKP</td>
<td>ECCE plus IOL</td>
<td>20/20 NA 1 41</td>
</tr>
<tr>
<td>2</td>
<td>HM</td>
<td>5</td>
<td>PKP</td>
<td>PEA plus IOL</td>
<td>20/25 Endothelial rejection (18 mo) 3 25</td>
</tr>
<tr>
<td>3</td>
<td>20/400</td>
<td>3</td>
<td>PKP</td>
<td>PEA plus IOL</td>
<td>20/32 Endothelial rejection (24 mo) 1 32</td>
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<td>4</td>
<td>20/1000</td>
<td>5</td>
<td>PKP</td>
<td>NA</td>
<td>20/100 NA 2 15</td>
</tr>
<tr>
<td>5</td>
<td>HM</td>
<td>11</td>
<td>PKP</td>
<td>PEA</td>
<td>20/500 CRVO 3 33</td>
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<tr>
<td>6</td>
<td>20/666</td>
<td>15</td>
<td>DLKP</td>
<td>PEA plus IOL</td>
<td>20/666 Haze 1 5</td>
</tr>
</tbody>
</table>

Abbreviations: BCVA, best-corrected visual acuity; CLET, cultivated limbal epithelial transplantation; CRVO, central retinal vein occlusion; DLKP, deep lamellar keratoplasty; ECCE, extracapsular lens extraction; HM, hand motions; IOL, intraocular lens implantation; KP, keratoplasty; NA, not applicable; PEA, phacoemulsification and aspiration; PKP, penetrating KP.

During the study, which spanned from September 1, 1999, to June 30, 2005, 26 CLETs were performed at the Department of Ophthalmology, Tokyo Dental College, on eyes with severe LSCD. Of these 26 eyes, 7 underwent subsequent keratoplasty. One eye was excluded because of unavailability for follow-up at 1 month, so 6 eyes were enrolled in this study, including 3 with alkali burns, 2 with Stevens-Johnson syndrome, and 1 with pseudo-ocular cicatricial pemphigoid.

Profiles of the patients are shown in Table 1. The patients ranged in age from 24 to 82 years (mean±SD age, 57.3±21.5 years). The corneal surface was covered by conjunctival epithelium in all patients, which was confirmed by slitlamp examination and impression cytology. Symblepharon was noted in 3 eyes, and all 6 eyes had reasonable reflex tearing with some tear meniscus. The mean±SD Schirmer value was 10.0±7.8 mm. All patients had a vascularization score of 4.

We used a 2-step surgical approach to treat LSCD: CLET to obtain a stable ocular surface in the first stage and optical keratoplasty in the second stage (Table 2). Donor limbal epithelium for CLET was obtained from the contralateral healthy eye in 1 patient, from the unaffected area of the same eye in 1 patient, from a living relative in 1 patient, and from nonrelated donor grafts in 3 patients. Following approval from the Institute Ethics Committee, written informed consent was obtained from all patients and donors after the purpose and potential risks of the procedures were explained. Clinical outcome was determined according to epithelial stability, neovascularization, graft clarity, and best-corrected visual acuity (BCVA). We used the neovascularization grading scale reported by Inatomi et al,15 which grades according to extent and intensity, with grade 1 indicating peripheral vascularization; grade 2, peripheral and midperipheral vascularization; grade 3, modest vascularization involving the entire cornea; and grade 4, massive vascularization of the entire cornea. Histopathological and phenotypic analyses of the epithelia before and following CLET were also reviewed.

**METHODS**

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**PREPARATION OF THE CLET SHEET**

In this study, we used the culturing method reported previously.16 In Brief, a limbal biopsy specimen of approximately 1×3 mm was obtained in the cases of auto-CLET and living-related CLET. In the allogeneic CLET cases, tissue from corneas obtained from the North West Lions Eyebank was excised to a similar size. The excised tissues were washed, cut into small pieces, and placed on a denuded AM substrate. Supplemental hormonal epithelial culture medium, including 5% fetal bovine serum or 5% of the patient’s serum, was used. When the epithelial culture reached confluence, stratification was induced by air exposure and mouse embryonic fibroblast cell line (American Type Culture Collection, Manassas, Virginia) for approximately 1 week.

**SURGICAL TECHNIQUES**

The fibrovascular pannus covering the cornea was excised at CLET and subjected to histopathological and phenotypic analyses. After release of the symblepharon and adequate homeostasis with cautery, subconjunctival application of 0.04% mitomycin for 3 minutes was performed, followed by vigorous
washing with 0.9% sterile saline. The CLET sheet was then transplanted onto the recipient’s cornea and sutured with 10-0 nylon and 8-0 polyglactin 910 (Vicryl). A bandage contact lens was applied to protect the ocular surface.

For optical purposes, keratoplasty was performed after a mean±SD interval of 6.8±5.1 months (range, 2-15 months) after CLET. The recipient cornea was excised using a disposable vacuum trephine with a 0.25-mm graft-host disparity. The excised corneal buttons were also subjected to histopathological and phenotypic analyses. The graft was secured with 10-0 nylon running sutures (extrainterrupted sutures were placed if necessary) and adjusted to reduce astigmatism. Either penetrating (n=5) or deep lamellar (n=1) keratoplasty was performed, depending on the depth of corneal opacification and involvement of the corneal endothelium. Intraocular lens insertion was also performed at the same time or as additional surgery, depending on the clinical situation in each case.

POSTOPERATIVE MANAGEMENT

After CLET, hyaluronic acid eyedrops (Hyalein Mini; Santen Pharmaceutical Co, Osaka, Japan), autologous serum eyedrops, and preservative-free corticosteroid eyedrops (Rinbeta PF; Nitten, Nagoya, Japan) were used for intensive epithelial maintenance. Antibiotics (ofloxacin [Tarivid] or levofloxacin [Cravit]; Santen Pharmaceutical Co, Osaka) and blood trough levels were maintained at 100 to 150 ng/mL, or at blood cyclosporine microemulsion levels 2 hours after a dose, 800 to 1000 ng/mL, unless abnormalities of renal and liver function developed. All patients also received systemic betamethasone sodium phosphate (Rinderon; Shionogi, Osaka) at an initial dosage of 8 mg, which was then tapered over 17 days. Although 2 patients underwent auto-CLET and theoretically did not require systemic immunosuppression, the same treatment was given because of severe neovascularization.

Following keratoplasty, the same continuous topical application of systemic immunosuppressant was performed for at least 6 months. When rejection developed, patients were treated with frequent topical corticosteroids combined with systemic corticosteroids.

ANTIBODIES

Mouse monoclonal antibodies for K3, cytokeratin 13 (K13), MUC5AC (Mucin 5 subtype AC), and α smooth muscle actin (SMA) were purchased from Affinity Research Products (AE5; Exeter, England), Ylem (Ks13.1; Rome, Italy), Biogenesis (2-12M1; Kingston, New Hampshire), and Sigma (1A4; St Louis, Missouri), respectively. Mouse IgG1 as a control was purchased from Dao Catenation (Lustrum, Denmark). Rhodamine-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania).

IMMUNOHISTOCHEMISTRY

Paraffin sections were deparaffinized in xylene and rehydrated. After antigen retrieval, we added protease XXV (Laboratory Vision Co, Fremont, California) at 37°C for 5 minutes before blocking. Sections were blocked by incubation with 10% normal donkey serum (Chemicon Int Inc, Temecula, California) and 1% bovine serum albumin (Sigma) for 1 hour. Antibodies to K3 (1:50), K13 (1:20), MUC5AC (1:100), and α-SMA (1:100) were applied and incubated for 90 minutes, followed by incubation with rhodamine-conjugated secondary anti-

body. After 3 washes with Tris-buffered saline with Tween 20, the sections were incubated with 1 mg/mL of 4',6-diamidino-2-phenylindole (Dijindo Laboratories, Tokyo) for 5 minutes. Finally, sections were washed 3 times in Tris-buffered saline with Tween 20 and a coverslip was placed using an antifading mounting medium.

RESULTS

CLINICAL OUTCOMES

Preoperatively, all the corneas were covered with highly vascularized conjunctiva and yielded a grade 4 neovascularization score. This score later decreased to grade 1 (3 eyes), grade 2 (1 eye), and grade 3 (2 eyes) after CLET (Table 1 and Table 2). Slitlamp examination together with fluorescein staining revealed that the ocular surface was covered in epithelia of the corneal phenotype. None of the eyes showed any major complications, such as microbial infection or secondary glaucoma, apart from 1 eye that showed a persistent epithelial defect, which later completely disappeared after topical medication (patient 2). No adverse effects were observed with the use of corticosteroids and cyclosporine. These 6 patients retained residual stromal opacity after CLET, which may inhibit improvement of visual acuity. Therefore, subsequent keratoplasty was scheduled in these patients.

After the subsequent keratoplasty, stable cornealike epithelia were maintained in all patients. The mean±SD follow-up was 25.1±13.2 months, and no repeated epithelial breakdown or conjunctivalization was observed. Although 2 eyes developed endothelial rejection 18 and 24 months after keratoplasty, both of them responded well to medical treatment and later regained clarity (Table 2).

Because of residual stromal opacification, post-CLET BCVA showed no significant (P=.30) improvement compared with preoperative BCVA. However, following keratoplasty, BCVA improved by more than 2 lines in all patients (range, 20/666-20/20) (Table 2). The BCVA was maintained until the last visit during the observation period. Limited visual improvement was observed in 2 eyes because of central retinal vein occlusion found after keratoplasty and interface haze after lamellar keratoplasty (Figure 1).

CASE REPORTS

Case 1

A 24-year-old man with severe corneal stromal opacity due to chronic phase alkali burns was seen by us in May 2002. His preoperative BCVA was 20/666. The ocular surface stabilized and vascularization and smooth epithelium showed a reduction following auto-CLET. However, preexisting stromal opacity complicated by a cataract limited the BCVA to 20/500. Two months after CLET, a combination of PKP and cataract surgery was performed to improve optical outcome. As a result, BCVA showed a significant improvement to 20/20, which was maintained for the succeeding 41-month period (Figure 2A-C).
Case 5

An 82-year-old woman with Stevens-Johnson syndrome that developed at the age of 28 years was referred to us in January 2001. Her BCVA was limited to counting fingers because of conjunctivalization and cataract. The ocular surface was reconstructed by living-related CLET. Postoperatively, smooth epithelialization was achieved. Although vascularization was reduced compared with preoperative vascularization, it nevertheless extended as far as the central cornea. Cataract surgery and PKP were performed at 6 and 11 months following CLET, respectively. Although the ocular surface remained stable throughout the 33-month follow-up following keratoplasty (Figure 2D-F), BCVA showed no recovery because of occlusion of the central retinal vein.

HISTOLOGICAL EXAMINATION

Corneal pannus specimens obtained at CLET revealed more than 10 layers of epithelia with chronic inflammatory cells in the vascularized stroma. Hematoxylin-eosin staining of the CLET sheet on the denuded AM revealed stratified epithelia throughout in all patients (data not shown). The corneal button obtained during keratoplasty revealed 5 to 7 layers of epithelium, with no inflammatory cells. Residual AM was found in the subepithelial region in 4 of the 6 patients (Figure 3).

IMMUNOHISTOCHEMISTRY

The results of histopathological examination are summarized in Table 3. The phenotypic study revealed that the epithelia in all specimens before CLET were positive for K13 (used as a conjunctival epithelial marker) but negative for the corneal-specific keratin, K3, indicating the conjunctival epithelial phenotype. The specimens obtained at keratoplasty revealed that the epithelial cells throughout the entire layer were positive for K3 but not for K13, in 4 of 6 patients, indicating the normal corneal epithelial phenotype (Figure 4).

Immunostaining against MUC5AC (used as a goblet cell marker) showed sporadically positive results, excluding 1 patient, before CLET, but totally negative results following CLET in all of the patients (Figure 5A-D). Furthermore, α-SMA–positive cells were observed in stromal cells before CLET, but no α-SMA–positive cells were observed following CLET (Figure 5E-H).

COMMENT

Penetrating keratoplasty has long been believed to be contraindicated in cases of eyes with severe LSCD arising from Stevens-Johnson syndrome, pseudo-ocular cicatricial pemphigoid, or alkali burns because the rate of failure was high. Although a combination of keratoplasty and limbal transplantation provides a stable epithelial surface for grafts, there is a greater risk of rejection. Recently, cultivated epithelial transplantation has been introduced to improve surgical outcomes in eyes with LSCD. Although the results have been encouraging, the effect of subsequent keratoplasty and its relationship with histological changes following epithelial transplantation have yet to be fully studied. In this study, post-CLET corneas revealed a decrease in neovascularization and inflammation in all patients, and the clinical outcome of subsequent keratoplasty was excellent (Table 2 and Figure 1). The results of histological analysis also revealed a decrease in neovascularization, inflammatory cells, expression of α-SMA in the stromal cells, and hyperplasia of the epithelium following CLET (Table 3 and Figure 3). Immunostaining to α-SMA was performed to determine the activity of stromal fibrosis and neovascularization, which was distinguished by vessel formation. Loss of α-SMA after CLET might be explained by the antiscarring effect of AM stroma.

These results indicate that CLET can bring about a recovery of epithelium of the corneal phenotype (ie, K3 positive, K13 negative, and MUC5AC negative), with this being demonstrated in 4 patients in this study, at least until keratoplasty (mean interval, 8.3 months). The clinical success of subsequent keratoplasty may be related to the recovery of the corneal phenotype, as Ti et al reported in a rabbit CLET model. Although restoration of the limbal phenotype and a normal basement membrane complex with laminin V have been reported in humans after ex vivo expanded limbal epithelial transplantation and keratolimbus allograft transplantation, in this study, 2 patients showed K13-positive, but K3-negative, epithelia (ie, the conjunctival phenotype) following CLET. These 2 patients also showed successful clinical outcomes following keratoplasty. Furthermore, both patients underwent allogeneic CLET and both did not have MUC5AC-expressing cells after CLET. Although the underlying mechanism is not clear, this may indicate that auto-CLET is superior to allogeneic CLET.
in terms of recovery of the corneal epithelium, a supposition supported by a study\textsuperscript{24} revealing that autograft showed a significantly higher ratio of graft survival than allograft in keratolimbal allograft. These successful outcomes may be attributable to the restoration of a microenvironment suitable for repopulation with the stem cells necessary for generation of corneal epithelium, a decrease in inflammation and neovascularization, and restoration of the basement membrane complex.\textsuperscript{24} Such a microenvironment has been reported to affect survival of limbal grafts in cases of eyelid abnormality, dry eye, and keratinization.\textsuperscript{24,25} Simultaneous surgery (PKP plus keratolimbal allograft) has a much worse graft survival ratio than 2-step surgery, which suggests that the microenvironment, especially in cases of severe inflammation, affects graft survival.\textsuperscript{26} Further study is necessary to determine whether the presence of corneal epithelium and its stem cells results in better long-term clinical outcomes. Two patients with phenotypical failure still had clinical success following PKP, which might be because the overall follow-up for them remained short (ie, 15 and 33 months). A long-term follow-up is neces-
necessary to address this important issue in allogeneic transplantation.

In an earlier study, the success rate of CLET in severe ocular surface disorders was reported to be 46.2%. In the present study, CLET was clinically successful in all 6 patients. This discrepancy may be because of selection bias; in this study, keratoplasty was performed only in clinically successful cases of CLET with residual corneal opacification. Success in ocular surface reconstruction following CLET may also be attributable to relatively good tear function, with + of 6 eyes showing a Schirmer value of greater than 5 mm. Recently, Inatomi et al reported on ocular surface reconstruction with the combination of cultivated autologous oral mucosal epithelial transplantation

**Table 3. Results of the Histopathological Examination**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of Epithelial Layers</th>
<th>Stromal Inflammatory Cells</th>
<th>K3</th>
<th>K13</th>
<th>MUC5AC</th>
<th>α-SMA</th>
<th>No. of Epithelial Layers</th>
<th>Stromal Inflammatory Cells</th>
<th>Residual AM</th>
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<th>K13</th>
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<tbody>
<tr>
<td>1</td>
<td>7-11</td>
<td>+++</td>
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<td>+++</td>
<td>+</td>
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<td>5-7</td>
<td>+</td>
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</table>

Abbreviations: AM, amniotic membrane; CLET, cultivated limbal epithelial transplantation; K3, cytokeratin 3; K13, cytokeratin 13; MUC5AC, Mucin 5 subtype AC; NA, not applicable; SMA, smooth muscle actin; +, low; ++, moderate; ++++, high; −, negative.

*a After CLET, MUC5AC and α-SMA were absent in all patients.

**Figure 4.** Cytokeratin 3 (K3) (A-F) and cytokeratin 13 (K13) (G-L) expression before cultivated limbal epithelial transplantation (CLET) (B, E, H, and K) and following CLET (C, F, I, and L). Images are shown for control conjunctiva (A and G), control cornea (D and J), patient 5 (B, C, H, and I), and patient 1 (E, F, K, and L). In patients 1 and 5, no K3 expression was observed in any epithelial layer, whereas K13 was positive in the suprabasal to superficial layers with hyperplasia (7-11 layers), before CLET. Following CLET, layers 5 to 7 expressed K3, but not K13, in patient 5, and K13 was only superficially positive in patient 1, suggesting that CLET allowed successful recovery of the corneal phenotype.
and PKP. The result also showed improving visual acuity and decreasing neovascularization. If autolimbal tissue is not available (ie, bilateral ocular surface diseases), not only allogeneic CLET but also autologous oral mucosal epithelial transplantation will be considered. There was no significant correlation between clinical outcome and duration between CLET and keratoplasty, the existence of AM following CLET, or original disease.

In summary, this study investigated the results of phenotypic analysis and clinical outcome in 6 cases of keratoplasty following CLET. Although the sample size was small, we believe that the excellent corneal graft survival rate and level of visual recovery achieved suggests a possible correlation with histological change following CLET. The results suggest that CLET restores a feasible microenvironment in the ocular surface and secures a corneal epithelial phenotype, which are important factors in the successful treatment of LSCD.

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