Original Article

Clinical, histological and immunohistochemistry characteristics of cornea in the sequelae stage of chronic vernal keratoconjunctivitis

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Purpose: To report the clinical outcomes and histopathological and immunohistochemistry (IHC) features in eyes with the sequelae stage of vernal keratoconjunctivitis (VKC). **Methods:** Investigative study of corneal samples obtained following surgical intervention for vision restoration in four eyes of three patients with VKC. Patient 1 (an 11-year-old boy) had deep anterior lamellar keratoplasty in both eyes, Patient 2 (a 24-year-old male) underwent superficial keratectomy followed by penetrating keratoplasty, and Patient 3 (a 22-year-old male) underwent penetrating keratoplasty. The corneal samples retrieved after surgical intervention were assessed for histology features and immunohistochemistry (IHC) studies. **Results:** The grafts were clear till the follow-up of 2–18 months. Histopathology of all four corneal samples showed epithelial hyperplasia, absent Bowman layer, thick hyalinized stromal lamellae, vascularization, and chronic inflammatory cells such as lymphocytes and plasma cells. IHC showed strong expression of CK 3 in both eyes of Patient 1 and no expression in Patients 2 and 3. The marker for limbal stem cells, ABCG2, was absent in all four samples; however, p63α was expressed strongly in Patients 2 and 3, moderately in the right eye of Patient 1, and marginally expressed in the left eye of Patient 1. **Conclusion:** The eyes in the sequelae stage of VKC (having corneal scarring and 360° hypertrophied limbus) can be managed favorably with keratoplasty and amniotic membrane transplantation without allogenic/cadaveric stem cell transplantation. The expression of transient progenitor cells in the scarred corneas of VKC patients in the sequelae stage suggests that the limbal stem cell dysfunction is more likely partial and self-renewal of limbal stem cells is a plausibility in these eyes.

Key words: Histopathology, immunohistochemistry, keratoplasty, limbal stem cells, vernal keratoconjunctivitis

Access this article online

Website: www.ijo.in

DOI: 10.4103/ijo.IJO_1179_21

Quick Response Code:

Vernal keratoconjunctivitis (VKC) is a chronic allergic condition that can lead to several ocular surface changes. Prolonged and recurrent allergic episodes over the years can progress to a sequelae stage of the disease characterized by scarring of conjunctiva and cornea, corneal vascularization, limbal hypertrophy, conjunctival pigmentation, and conjunctivalization of the cornea, even though the acute allergic episodes may have subsided.[1-5]

In view of dense corneal vascularization and the likelihood of limbal stem cell dysfunction because of chronic limbal inflammatory episodes in the past, keratoplasty in the sequelae stage of VKC may not be an easy decision to make.[6-8] It is unclear if the damage to the limbal stem cell niche is partial or complete in the sequelae stage of the disease. A complete deficiency of limbal stem cell niche would warrant an allogenic stem cell transplant along with keratoplasty, which necessitates immunosuppressants in the post keratoplasty management. We have observed a subset of patients in the sequelae stage of VKC with severe limbal hypertrophy 360°, who are often seen with clear central corneas (*as shown in* Fig. 1). In view of this clinical presentation in some eyes, it is hypothesized that the stem cell deficiency resulting in this condition is more likely partial or possibly the limbal stem cell function is suppressed.

There is limited literature on management guidelines of visual rehabilitation of eyes with extensive corneal scarring with the hypertrophic limbal region and the outcomes of corneal transplant in end/burnt-out stage of VKC is not very well reported. The purpose of this study is to report the outcomes of surgical intervention and to characterize the histological and immunohistochemistry features of corneal samples of patients with VKC in the sequelae stage who underwent keratoplasty.

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Received: 12-May-2021 Revision: 13-Jul-2021
Accepted: 06-Aug-2021 Published: 23-Dec-2021

Cite this article as: Jaffet J, Singh V, Chaurasia S, Jakati S, Hazari A, Sangwan V. Clinical, histological and immunohistochemistry characteristics of cornea in the sequelae stage of chronic vernal keratoconjunctivitis. Indian J Ophthalmol 2022;70:59-64.

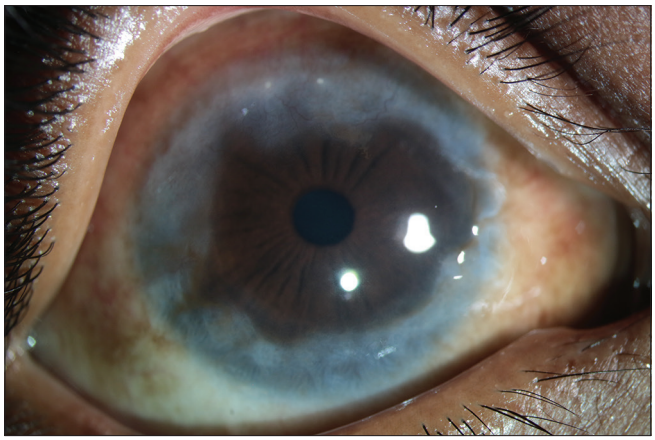

Figure 1: Representative photograph of the right eye of a patient with chronic VKC showing hypertrophic and scarred limbal region with clear central cornea

Methods

The study included four eyes of three patients, who were clinically diagnosed with the sequelae stage of VKC (defined as having corneal scarring and 360° hypertrophied limbus after symptoms of active allergy have subsided). The study was conducted in accordance with the tenets of the Declaration of Helsinki and informed written consent from patients and the attendants. Two samples of one patient (Patient 1) were obtained after deep anterior lamellar keratoplasty, one after superficial keratectomy (Patient 2) and one after penetrating keratoplasty (Patient 3). The corneal buttons were bisected carefully and processed. Five-micron-thick sections of formalin-fixed paraffin-embedded tissues were stained with Hematoxylin and Eosin (H and E) and Periodic Acid Schiff stains. Immunohistochemistry (IHC) was performed on corneal buttons of four eyes of the three patients as per the procedure described earlier.[9] All the samples were stained for corneal epithelium marker (CK3), conjunctival marker (CK19), limbal stem cell marker (p63alpha and ABCG2). Briefly, the procedure involved the following steps:

Deparaffinization and antigen retrieval: Slides with sections were heated in a heat block at 70°C for 3 min. Deparaffinized slides were dipped in three exchanges of xylene, for 5 min each. The sections were hydrated with serial dilution of 100%, 90%, and 80% ethanol, respectively, in a rocker for 5 min each. Finally, sections were rinsed in distilled water before antigen retrieval. Preheating of the Coplin jar containing citrate buffer (pH = 6) was performed until the temperature reached 95°–100°C. The slides were immersed in the Coplin jar and heated for 15 min. After 15 min, these were allowed to cool down to room temperature.

Permeabilization and Blocking: Sections were washed with 1X phosphate buffer saline (PBS) and treated with 0.5% Triton X-100 for 30 min. After incubation, sections were washed with 1X PBS and blocking was done with 2.5% bovine serum albumin (BSA) for 45 min.

Staining with Antibodies: The tissue was treated with a dilution of primary antibody (Supplementary Table 1) and incubated overnight at 4°C in a moist chamber. After appropriate washing with 1X PBS, the tissue sections were treated with corresponding secondary antibodies in dark conditions and incubated for 45 min in a moist chamber. Antibody dilutions were prepared using 1% BSA and 4′,6-Diamidino-2-phenylindole DAPI was used for nuclear staining.

This is an observational and retrospective study after ethics committee approval.

Results

Fig. 2 shows the clinical slit-lamp photographs of the three patients. Figs. 3 and 4 show the spectrum of histopathological findings and immunohistochemistry images of the four samples, respectively. Table 1 summarizes the histology and immunohistochemistry characteristics.

Surgical technique and postoperative management: The surgery was performed under general anesthesia in one patient (Patient 1) and local anesthesia in the other two patients (Patients 2 and 3). The center of the cornea was identified and keratoplasty was performed with a donor size of 8.25 mm using 16 interrupted 10-0 nylon sutures. A limited superior 6–7 clock hours of peritomy was performed 3 mm away from the limbal region. Gentle cautery was applied to the bleeding vessels. This was followed by the amniotic membrane transplantation using fibrin glue in the manner of a doughnut technique[7] as described earlier. Briefly, the amniotic membrane with a 4–5 mm window in the center (created either freehand or with a trephine) was applied on the corneal surface to leave the central visual axis clear in the postoperative period. The amniotic membrane was tucked beneath the conjunctiva superiorly. A bandage contact lens was applied, and lateral permanent tarsorrhaphy was performed to retain the amniotic membrane and improve ocular surface health. The bandage contact lens was removed at approximately 3–4 weeks and tarsorrhaphy was released at 3 months after the surgery. Postoperative care involved topical steroids in tapering doses, topical antibiotics until bandage contact lens application, and oral immunosuppressants under the supervision of internists.

A. Clinical features and outcomes after corneal surgery

Patient 1: An 11-year-old boy presented with a history of recurrent episodes of itching and redness in both eyes from 5 years of age. At presentation, he revealed that there were no complaints of itching in the eyes since the past 1 year, but his vision had deteriorated severely over the past several months. His visual acuity was counting fingers at 1 m in both eyes. On slit-lamp examination, the limbal region was hypertrophic, densely hyperpigmented, tarsal conjunctiva was flat and pigmented, and the cornea was scarred and vascularized [Fig. 2a and b]. B scan ultrasonography was echo-free with no evidence of optic nerve cupping. Intraocular pressure (IOP) was in the normal range in both eyes with an iCare tonometer. The patient underwent deep anterior lamellar keratoplasty along with amniotic membrane transplantation with simultaneous lateral tarsorrhaphy in the left eye followed by the right eye at an interval of 6 weeks apart [Fig. 2c and d]. The surgery involved dissection up to the pre-descemetic plane with suturing of the donor cornea using 16 interrupted 10-0 nylon sutures. This was followed by the amniotic membrane transplantation, application of a bandage contact lens, and lateral permanent tarsorrhaphy. The lamellar corneal samples retrieved after keratoplasty were subjected to histology and IHC examination. The post keratoplasty treatment

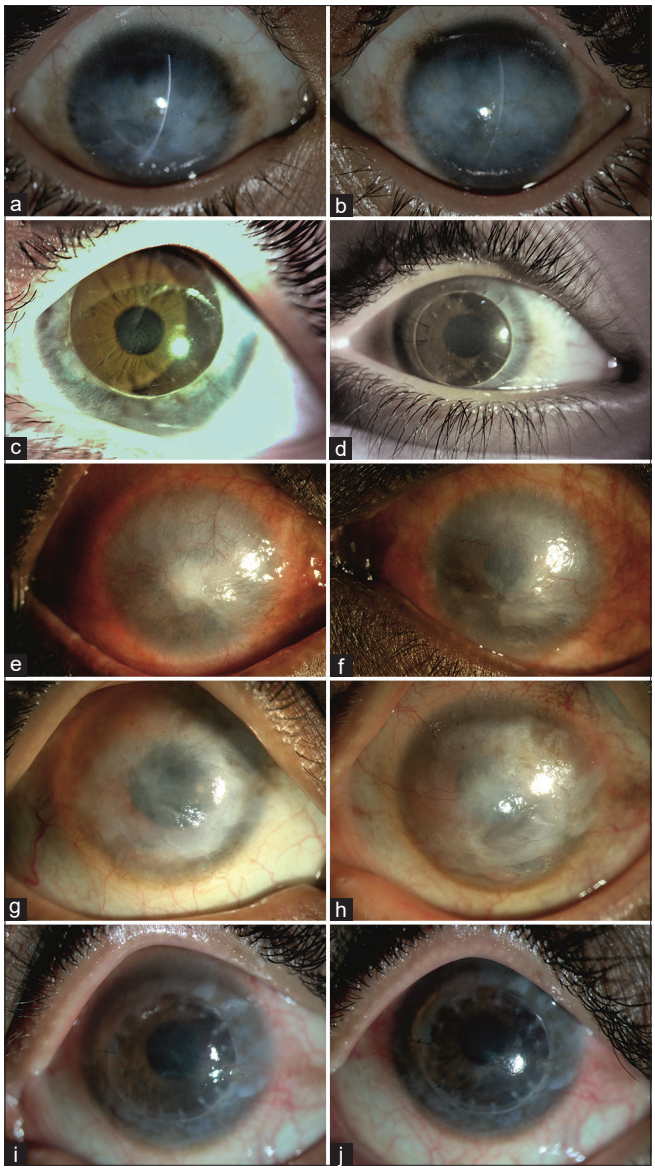

Figure 2: Pre-operative (a and b) and postoperative (c and d) images of Patient 1, after deep anterior lamellar keratoplasty in the right and left eye, respectively; Slit-lamp pictures of the right and left eye (e and f) of Patient 2; Slit-lamp photographs of right (g) and the left eye (h) of the Patient 3. Left eye that underwent penetrating keratoplasty presented with broken sutures and inflamed ocular surface (i) at 1.5-year follow-up, which resolved well after suture removal and steroid treatment (j)

involved broad-spectrum antibiotics for 1 week, tapering doses of prednisolone acetate 1% at least twice daily, and tacrolimus 0.3% ointment at bedtime in both eyes. The patient was advised oral methotrexate 5 mg weekly for 3 months. Postoperatively, the visual acuity improved to 20/50 in the left eye and 20/100 in the right eye. Suture removal was performed when loosening of sutures was noted. The bandage contact lens was kept in place for 4 weeks and tarsorrhaphy was released at 3 months. The grafts were clear, visual acuity improved and maintained at 20/40 in both eyes, and IOP was normal till the last follow-up of 1.5 years.

Patient 2: A 24-year-old male presented with poor vision in both eyes for 6 years. He had previously suffered recurrent episodes of itching and redness in his eyes since early childhood.

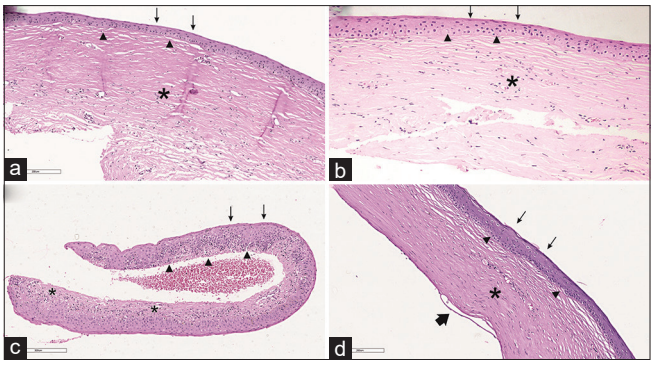

Figure 3: Photomicrographs of the keratoplasty specimens show a and (b) Patient 1- Mild epithelial hyperplasia (arrows), absent Bowman layer (arrowheads), thickened and vascularized stroma with myofibroblastic transformation (asterix). Scattered chronic inflammatory cells are seen. (Hematoxylin and Eosin; ×100); (c) Superficial keratectomy specimen shows epithelial hyperplasia with intraepithelial lymphocytes (arrows), absent Bowman layer (arrowheads), and thickened stroma with chronic inflammatory cells (asterix) (Hematoxylin and Eosin; ×100); (d) Focal epithelial hyperplasia with mild keratinization (arrows), absent Bowman's layer (arrowheads), stroma is thickened, hyalinized with vascularization and perivascular infiltrates (asterix). Descemet membrane is focally detached (bold arrow) with occasional endothelial cells (Hematoxylin and Eosin; ×100)

At this visit, his visual acuity was counting fingers 1 m in the right eye and 20/100 in the left eye. Slit-lamp examination revealed flat scarred tarsal conjunctiva, limbal hypertrophy, pigmentation, corneal scarring with 360° vascularization in both eyes [Fig. 2e and f]. Lens appeared clear through the hazy corneas. He underwent superficial keratectomy with amniotic membrane transplantation, bandage contact lens application, and lateral permanent tarsorrhaphy in the right eye. The corneal sample after superficial keratoplasty was sent for histology analysis. He was treated with weekly tapering doses of prednisolone acetate 1% and preservative-free lubricants. The postoperative course was uneventful and visual acuity improved to 20/100. He was explained the option of prosthetic replacement of the ocular surface ecosystem (PROSE) trial, which he was not keen on. Subsequently, he underwent penetrating keratoplasty after 6 months, which further improved his vision to 20/50. Following keratoplasty, he was treated with prednisolone acetate 1% eyedrops, tacrolimus 0.1% eye ointment twice daily, and oral azathioprine 50 mg twice daily, which he was advised to continue for the next 6 months under the supervision of an internist. His visual acuity was maintained till the last follow-up of 2 months, beyond which he was on follow-up at his local place. The corneal button of this patient after penetrating keratoplasty was unavailable for further histological analysis.

Patient 3: A 22-year-old male presented with complaints of poor vision in both eyes for the past 15 years. He had suffered from recurrent severe allergy in both eyes as per his previous medical records. At this visit, the visual acuity was 20/200 in the right eye and counting fingers 1 m in the left eye. The limbal region was hypertrophic with increased pigmentation, and the cornea showed scarring with vascularization in both eyes [Fig. 2g and h]. He was not keen on opting for a PROSE trial in the right eye. He underwent penetrating keratoplasty in the left eye with a doughnut amniotic membrane

Table 1: Histopathological features and IHC of the corneal samples: The corneal epithelial biomarker CK3 was strongly represented in both eyes of Patient 1. The conjunctival biomarker CK 19 was seen to be moderately positive in the right eye of Patient 1 and Patient 3. The limbal stem cell markers were tested negative in all samples. The marker for transient amplifying cells were strongly expressed in two samples (Patient 1 right eye, Patient 3), moderately expressed in 1 (right eye of Patient 2), and mildly expressed in one sample of Patient 1 left eye)

Patient No.	Eye	Specimen	Epithelium	Bowman's layer	Stroma	Descemet membrane	Endothelium	CK3	CK19	ABCG2	P63α
Patient 1	OD	Deep Lamellar Cornea	Epithelial hyperplasia	Absent	Lamellae thick and hyalinized, vascularization, chronic inflammatory cells	Not present	Not present	+++	++	-	+++
	OS	Deep Lamellar cornea	Epithelial hyperplasia	Absent replaced by degenerative pannus	Anterior stroma thick and hyalinized collagen, Vascularization with perivascular infiltrates	Not present	Not present	+++	-	-	+
Patient 2	OD	Superficial lamellar cornea	Epithelial hyperplasia with elongated rete pegs	Absent replaced by degenerative pannus	Thick and hyalinized collagen lamellae with scattered infiltrate.	Not present	Not present	-	-	-	++
Patient 3	OS	Full-thickness cornea	Epithelial hyperplasia	Absent	Thick and hyalinized lamellae with perivascular infiltrates. Thickened blood vessels	Normal and focally detached	Occasional Endothelial cells	-	++	-	+++

"-" No expression of the given marker. "+" Low expression of given marker (<25% in total cell population). "++" Moderate expression of the given marker (between 25%-75% in total cell population). "+++" Very high expression of given marker (more than 75% in total cell population)

transplantation, bandage contact lens application, and lateral permanent tarsorrhaphy. The corneal button was subjected to histology and IHC examination. His postoperative course was uneventful. He was treated with oral methotrexate of 7.5 mg/weekly and tapering doses of steroids twice daily and reviewed periodically in his native country. He presented to our clinic 1.5 years after keratoplasty, when he had several broken exposed sutures, with vascularization at the site of suture beds [Fig. 2i and j]. The sutures were removed and prednisolone acetate 1% was advised every 3 hours. At 1-week post suture removal, the eye was quiet, the graft was clear, and visual acuity had improved to 20/80.

B. Histological features

The histopathological features are summarized in Fig. 3 and Table 1. In all four samples, the epithelium showed hyperplasia and the Bowman's layer was absent. The stromal lamellae were thick, hyalinized, and showed chronic inflammatory cells (lymphocytes and plasma cells). Eosinophils were seen in the stroma of the sample obtained from Patient 2. Vascularization was noted in all four samples. Descemet membrane and endothelium were normal in one sample (full thickness) where it was available for analysis.

C. Analysis of epithelium and stem cell markers using immunofluorescence

The corneal epithelial biomarker (CK3) was expressed strongly in both eyes of Patient 1 and was lacking in Patients 2 and 3. The conjunctival biomarker (CK19) was moderately expressed in the right eye of Patient 1 and the left eye of Patient 4. All four samples showed an absence of immunostaining with stem cells marker ABCG2. However, of the four samples, three [OD of Patient 1 (OS had a low expression), Patient 3, and Patient 4] revealed a strong presence of progenitor stem cells marker (p63 alpha). The quantitative analysis for the expression of these markers is depicted in Fig. 4.

Discussion

The patients described in the study included those in the sequelae stage of the chronic VKC which is characterized by ocular surface changes and absence of symptoms of acute allergic episodes such as itching and tearing from the eyes. The resultant corneal scarring severely compromises the visual acuity in these patients; hence, surgical intervention to restore corneal clarity is required. The outcomes after deep lamellar keratoplasty in two eyes one patient (Patient 1), penetrating keratoplasty in two eyes of two patients (Patients 2 and 3) was favorable till the last follow-up range of 2–18 months. In one patient (Patient 3), at the last follow-up period, the sutures were broken with exposed ends that led to inflamed ocular surface, which recovered well after suture management. Amniotic membrane is well known to have a beneficial role in promoting and maintaining ocular surface[9] and hence, surgical intervention in all four eyes involved additionally amniotic membrane transplantation with bandage contact lens application and a lateral paramedian tarsorrhaphy.

In a study that involved impression cytology of conjunctiva and cornea in eyes with VKC associated with clinical features suggestive of limbal stem cell deficiency, goblet cells were found in 53.6% of the corneal samples.[10] In the samples tested in our study, goblet cells could not be identified. The histopathological features of the excised pannus and corneal specimen in VKC patient has shown hyperplastic epithelium, absent Bowman layer, disorganized lamellar architecture, mixed inflammatory cells, lymphoplasmacytic infiltration, blood vessels in the stroma, and fragmented Descemet membrane with reduced endothelial cells.[7] The histopathological features of the samples in this study were consistent with the features reported earlier and included epithelial changes such as hyperplasia and absent Bowman layer. The epithelial layer was better organized in three samples compared to the sample studied from Patient

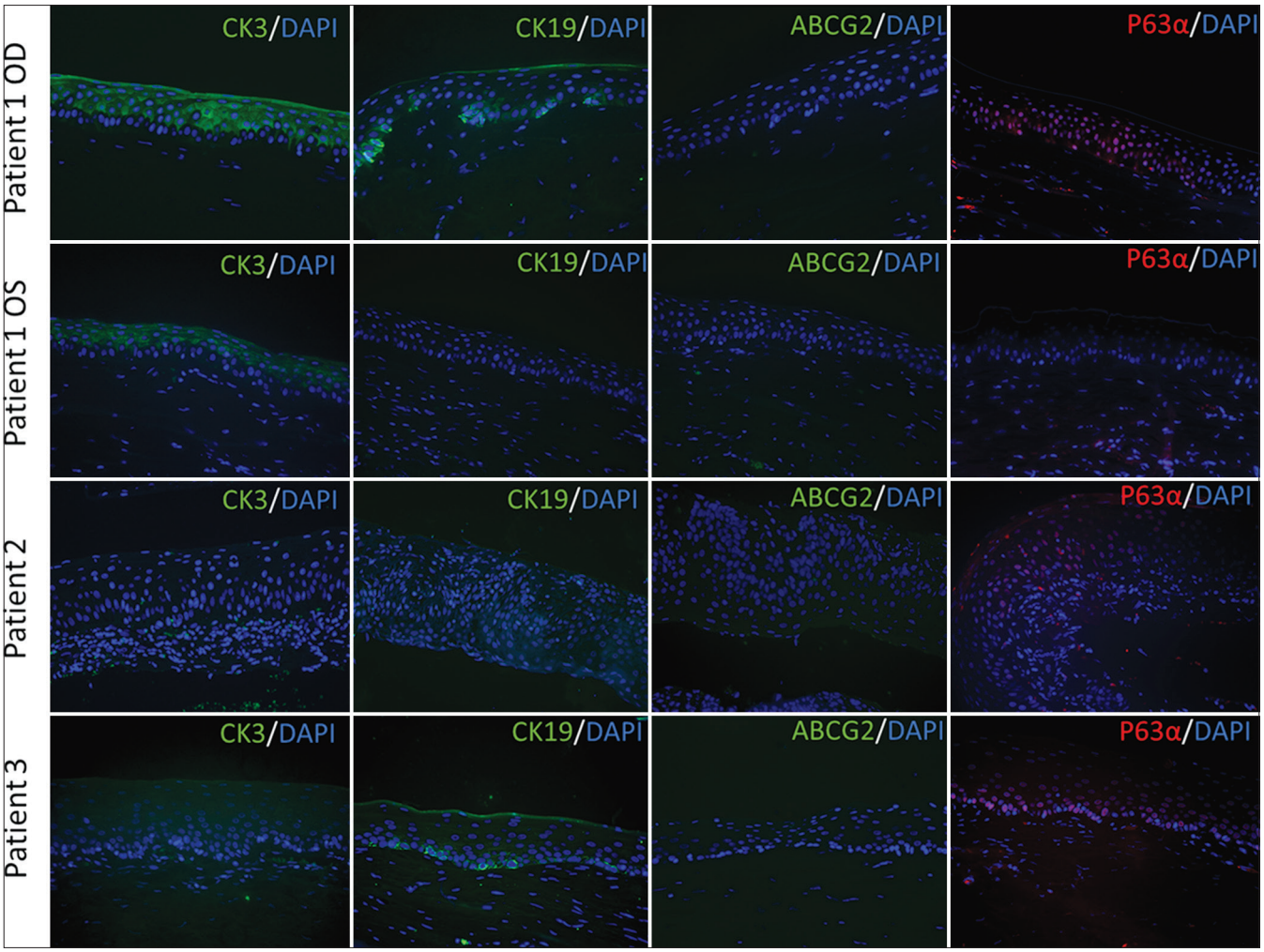

Figure 4: The localization of CK3 (panel one: green), CK 19 (panel two: green), ABCG2 (panel three: Green), P63α (panel four: red), in tissue sections of the four eyes. (CK19 is a marker specific for conjunctival epithelium, ABCG2 is a universal marker for stemness, P63α is a limbal stem cell marker and CK3 is a corneal epithelium-specific marker. DAPI- Blue stain showing nucleus)

2 where it showed severe disorganization and elongation of rete pegs. This correlates with the severe epithelial irregularity that was also clinically apparent in this patient. The stromal lamellae were thick, hyalinized, and invaded with chronic inflammatory cells. All eyes had vascularization on clinical slit-lamp evaluation, which was consistent in the histological analysis.

The IHC showed a strong expression of CK3 in both eyes of Patient 1, whereas it was lacking in Patient 2 and Patient 3. The IHC showed absence of limbal stem cell marker ABCG2 in all specimens, but the marker for transient amplifying cells p63 alpha was expressed to variable extents in the four samples. The IHC markers ABCG2 and p63alpha are routinely used to identify limbal stem cells or transient amplifying cells respectively of the limbus or even other stem cells. These are the hallmark markers for identifying any stem cells. However, CK3 is a marker to identify corneal epithelial cells specifically and CK19 is used to identify conjunctival epithelial cells exclusively.[11,12] The absence of limbal stem cell markers could be because the samples were obtained from the central 7.75 mm of the cornea (keratoplasty samples) without encroaching on to the limbal region. However, the presence of progenitor cells is a noteworthy finding as it implies that there is some expression of stem cell activity, which may have the capability to restore limbal stem cell functionality even in the sequelae stage of the disease. It is hypothesized that the stem cell niche may be suppressed/dysfunctional in the various stages of the disease and not completely deficient. This plausibility can explain the favorable graft survival in the four eyes of three patients without the application of allogenic/cadaveric stem cell transplantation in these eyes and the clinical observation of clear central cornea despite severe limbal hypertrophic changes in some eyes with chronic and recurrent VKC. In our recent study[13] in the rabbit limbal stem cell deficiency model, we observed that despite all clinical features suggestive of limbal stem cell deficiency for several months, the limbal region showed self-renewal without any surgical intervention, lending support to the observations discussed in this study.

The findings and conjecture proposed in this study need further validation in a greater number of patients with a similar spectrum of the disease. Further, the samples from the limbal region could be studied to have a better insight into the limbal stem cell biomarkers. If these observations are reproducible in larger studies, it would help in formulating an algorithm of

management of VKC patients with this spectrum of corneal involvement.

Conclusion

The eyes in the sequelae stage of VKC can be managed favorably with keratoplasty, amniotic membrane transplantation and tarsorrhaphy. The expression of transient progenitor cells in the scarred corneas of VKC patients in the sequalae stage is a noteworthy finding in the study. Low dose immunosuppression enhances the chances of graft survival in this high risk situation.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: List and details of primary and Secondary antibodies used and their dilutions details

Primary/Secondary Antibody	Dilution	Catalog No.	Company
CK19	1:100	SC376126	Santa Cruz Biotechnology
ABCG2	1:100	SC18841	Santa Cruz Biotechnology
P63α	1:100	CST4892	Cell Signaling Technology
CK3	1:100	ab68260	Abcam
Alexa Fluor 488, Anti mouse	1:400	A11001	Invitrogen
Alexa Fluor 594, Anti Rabbit	1:400	A11012	Invitrogen