Limbal Stem Cell Deficiency and Ocular Phenotype in Ectrodactyly-Ectodermal Dysplasia-Clefting Syndrome Caused by *p63* Mutations

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Objective: To describe the ocular phenotype in patients with ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome (MIM#604292) and to determine the pathogenic basis of visual morbidity.

Design: Retrospective case series.

Participants: Nineteen families (23 patients) affected by EEC syndrome from the United Kingdom, Ireland, and Italy.

Methods: General medical examination to fulfill the diagnostic criteria for EEC syndrome and determine the phenotypic severity. Mutational analysis of *p63* was performed by polymerase chain reaction–based bidirectional Sanger sequencing. All patients with EEC syndrome underwent a complete ophthalmic examination and ocular surface assessment. Limbal stem cell deficiency (LSCD) was diagnosed clinically on the basis of corneal conjunctivalization and anatomy of the limbal palisades of Vogt. Impression cytology using immunofluorescent antibodies was performed in 1 individual. Histologic and immunohistochemical analyses were performed on a corneal button and corneal pannus from 2 EEC patients.

Main Outcome Measures: The EEC syndrome phenotypic severity (EEC score), best-corrected Snellen visual acuity (decimal fraction), slit-lamp biomicroscopy, tear function index, tear breakup time, LSCD, *p63* DNA sequence variants, impression cytology, and corneal histopathology.

Results: Eleven heterozygous missense mutations in the DNA binding domain of *p63* were identified in all patients with EEC syndrome. All patients had ocular involvement and the commonest was an anomaly of the meibomian glands and lacrimal drainage system defects. The major cause of visual morbidity was progressive LSCD, which was detected in 61% (14/23). Limbal stem cell deficiency was related to advancing age and caused a progressive keratopathy, resulting in a dense vascularized corneal pannus, and eventually leading to visual impairment. Histologic analysis and impression cytology confirmed LSCD.

Conclusions: Heterozygous *p63* mutations cause the EEC syndrome and result in visual impairment owing to progressive LSCD. There was no relationship of limbal stem cell failure with the severity of EEC syndrome, as classified by the EEC score, or the underlying molecular defect in *p63*.

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Ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome (MIM#604292) is an autosomal-dominant disorder with highly variable expression and reduced penetrance, resulting in a marked intrafamilial and interfamilial variability.[1–3] The phenotypic spectrum and variable expressivity make clinical diagnosis and classification difficult.[2,4] Celli et al[4] mapped a locus for EEC syndrome to a genomic interval on chromosome 3q27 and subsequently identified mutations in *p63* in patients with EEC syndrome. The cloning and identification of *p63* as the causative gene for the EEC syndrome has allowed an objective molecular diagnosis and provides a basis to explore the pathogenesis of the ocular phenotype of EEC syndrome. Ocular features are more commonly reported in EEC syndrome when compared with the other ectodermal dysplasias, and many authors include lacrimal drainage anomalies as one of the cardinal signs of the syndrome.[2,3] Few reports have focused on the progressive keratopathy in EEC syndrome. Recurrent corneal epithelial defects, corneal ulceration, corneal pannus and neovascularization, and corneal thinning and scarring have been reported.[3,5–8]

The pathogenesis of the corneal disease is unclear. Some authors have suggested that recurrent lacrimal infection,[9,10] dry eye disease,[11] and trichiasis account for the corneal features, whereas others have viewed the keratopathy in EEC syndrome as a primary manifestation of the ectodermal dysplasia itself.[6,12,13] The pathogenesis of the ocular phenotype in EEC syndrome has been poorly described in the

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literature owing to deficiencies in understanding the molecular basis of EEC syndrome. This article describes the ocular phenotype in a molecularly characterized cohort of patients with EEC syndrome and establishes limbal stem cell deficiency (LSCD) as the major cause of ocular morbidity.

Materials and Methods

Human Subjects

Patients with EEC syndrome were recruited from the United Kingdom Ectodermal Dysplasia Society (http://www.ectodermaldysplasia.org/; accessed February 17, 2011); the 2 Italian EEC societies (http://www.sindrome-eec.it/ and http://www.aieec.it/; both accessed February 17, 2011); and referral from clinicians. All applicable institutional and governmental regulations concerning the ethical involvement of human volunteers were observed during this research. The study was approved by the Liverpool Research and Ethics Committee (UK) and the Multiregional Research and Ethics Committee (North-West, UK) and the Venetian Clinical Research Ethics Committee (Venice, Italy). After obtaining consent, full medical and ocular histories were taken and a complete family history was recorded to document the pedigree. The clinical diagnostic criterion for EEC syndrome was that all EEC families or isolated cases had ≥2 of the 3 main features of the syndrome[3,14]: (1) An ectodermal dysplasia affecting the skin, hair, nails, teeth, sweat glands, lacrimal ducts, or mammary glands; (2) hand or foot abnormalities consistent with the split hand–split foot spectrum; and (3) cleft lip with or without cleft palate. To quantify the phenotypic severity of EEC syndrome a previously published scoring system was used.[1] This system scores the severity of ectodermal dysplasia, ectrodactyly, clefting, and associated features with a maximum total score of 18, indicating a severe EEC phenotype.[1]

The ocular assessment included best-corrected Snellen visual acuity (decimal fraction), slit-lamp biomicroscopic evaluation, and ocular surface assessment. To evaluate the ocular surface and tear dynamics a combination of Schirmer testing, measurement of tear function index,[15] tear meniscus height, tear film breakup time and fluorescein staining were performed. Specifically, the height of the inferior and superior tear menisci (<0.2, 0.2–0.4, and >0.4 mm) were recorded using the 0.2-mm beam of the slit lamp,[16] and the tear film breakup time was calculated as the time between last blink and first disturbance of the corneal tear film, with tear film instability indicated by a breakup time of <10 seconds.[16,17] Nasolacrimal duct function (dye disappearance testing and testing for patency) was performed when appropriate. If present, cicatricial conjunctivitis was graded using a modification of the grading system for ocular involvement in mucous membrane pemphigoid[18] (Table 1). Corneal findings were recorded in a standard fashion with color-coded frontal and slit-beam sketches[19] and with anterior segment photography. Clinically, LSCD was diagnosed on the basis of well-established and reliable criteria[20]: (1) corneal conjunctivalization producing fibrovascular tissue (pannus), (2) irregularity and permeability of corneal epithelium (revealed by fluorescein staining), and (3) anatomy of the limbal palisades of Vogt. Impression cytology was performed in 1 individual using immunofluorescent antibodies against keratin (K) 12 (sc-17099, goat polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and mucin (MUC) 1 (H-295; sc-15333, rabbit polyclonal, 1:200, Santa Cruz Biotechnology), which can discriminate corneal epithelia from conjunctival epithelia, respectively.[21]

Human Samples

Three different types of human samples were obtained from EEC patients. A 10-ml sample of venous blood in ethylenediaminetetraacetic acid (EDTA) was taken from all patients by sterile venipuncture. Genomic DNA was isolated from peripheral blood leukocytes using standard protocols (Wizard DNA purification kit; Promega, Madison, WI). A corneal button was obtained for histologic analysis in 1 affected patient (EEC010-1) who underwent penetrating keratoplasty (PK) for visually significant corneal stromal scarring. A fibrovascular pannus excised from a 48-year-old subject (EEC014-1) undergoing ocular surface reconstruction (symblepharon lysis, fornix reconstruction by amniotic membrane transplantation, and superficial keratectomy) was available for histology and immunohistochemistry. The sample was fixed in 4% paraformaldehyde (overnight at 4°C), embedded in optimal cutting temperature compound, frozen, and sectioned. Sections (5–7 μm) were analyzed by indirect immunofluorescence.

Immunofluorescence

Immunofluorescence studies were performed by using antibodies against K12 (sc-17099, goat polyclonal, 1:100, Santa Cruz Biotechnology); MUC1 (H-295; sc-15333, rabbit polyclonal, 1:200, Santa Cruz Biotechnology); K19 (RB-9021, rabbit polyclonal, 1:200, NeoMarkers, Freemont, CA); pan-p63 (4A4, mouse monoclonal, 1:100; BD Biosciences, Milan, Italy); p63ΔNα (rabbit polyclonal, 1:200; PRIMM, Milan, Italy); CD-45 (555480, mouse monoclonal, 1:100; BD Biosciences); MUC4 (sc-20117, rabbit polyclonal, 1:100; Santa Cruz Biotechnology) for 1 hour at 37°C. Rhodamine and fluorescein isothiocyanate-conjugated secondary antibodies (1:100; Santa Cruz Biotechnology) were incubated for 1 hour at room temperature. Specimens were analyzed with an LSM 510 Meta Confocal Microscope (Zeiss SpA, Milan, Italy).

Mutation Analysis of the *p63* Gene

Genomic DNA was used as a template for amplification of *p63* DNA-binding domain (exons 4–8) genomic sequences, as previously described.[4,14] Amplified DNA fragments were sequenced using both forward and reverse primers and analyzed on ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Identified sequence variants were described according to the guidelines published by Human Genome Variation Society (http://www.hgvs.org/mutnomen/; accessed February 17, 2011). The position of mutations is given according to the original published TA-*p63α* sequence (GenBank accession no. AF075430), which does not encode the 39 additional amino acids at the *N*-terminus as reported by Hagiwara et al[22] (GenBank accession no. AF091627; gi: 3695081). Mutation positions were therefore based on nucleotide numbering of the *p63α* isotype with the A of the methionine start codon as 1. To exclude polymorphisms, a panel of 100 ethnically matched control individuals (200 chromosomes) was screened for any identified sequence variations and segregation was assessed within the family if available. Sequence changes were also compared with previously reported *p63* mutations,[14,23,24] and the functional effects of nonsynonymous sequence variants that were absent from the control population were predicted using a range of freely available computational tools: ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), PolyPhen (*Poly*morphism *Phen*otyping: http://genetics.bwh.harvard.edu/pph/), PMut (http://mmb2.pcb.ub.es:8080/PMut/), and SIFT (http://sift.jcvi.org/; all websites accessed February 17, 2011).

Results

Mutation Analysis of the *p63* Gene

Nineteen families consisting of 23 patients with EEC syndrome, diagnosed clinically, were screened for mutations in *p63*. Sequence changes were detected in all families tested and consisted of eleven separate missense nucleotide substitution mutations (Table 1). Ten mutations were previously reported in *p63*; a novel, previously unreported mutation (p.S272T) was detected in 2 unrelated Italian EEC patients. All mutations were in the DNA-binding domain of *p63* that extends from exons 4 to 8. No other potentially pathogenic sequence variants were identified in the other exons or flanking intronic sequences of *p63*. The mutations were not seen in unaffected family members, when DNA was available, or in 200 control chromosomes. Residue R304 and, to a lesser extent, R279 were mutational hotspots, representing 31.6% (6/19) and 26.3% (5/19) of the identified mutations in this cohort, respectively. Mutations involved an arginine residue in 73.7% (14/19) of cases. There was no relationship between the clinical severity of EEC syndrome,[1] as classified by EEC syndrome score, and the underlying molecular defect in *p63* (Table 1).

Ocular Phenotype of the EEC Syndrome

Twenty-three patients had a full ocular assessment; the ocular phenotype is summarized in Table 1 and the typical clinical features are shown in Figure 1. All examined patients had ocular defects, and the commonest was an anomaly of the meibomian glands (Fig 1B), which was present in all 23 cases (100%). Meibomian gland defects resulted in an unstable tear film, as measured by the tear film breakup time, and an evaporative dry eye in all patients in whom tear dynamics could be assessed. Lacrimal drainage system defects (absence, occlusion, or stenosis) present in 91.3% (21/23) of cases were the second commonest ocular anomaly. Aqueous tear deficiency, as measured by the tear meniscus height and tear function index (TFI) was present in 56.5% (13/23) of cases. Conjunctival cicatrization was seen in 39.1% (9/23) of patients (Fig 1B, D).

LSCD Is the Major Cause of Visual Morbidity in Patients with EEC Syndrome

The major cause of visual morbidity was LSCD, which was seen in 60.9% (14/23) of cases (Table 1; Fig 1G, I; Fig 2A). All patients with LSCD had an absence of the limbal palisades (i.e., the niche of corneal stem cells). Complete absence of the limbal palisades was seen in 65.2% (15/23) and defective palisade anatomy in 26.1% (6/23) of cases. Impression cytology specimens collected from a 48-year-old EEC woman (EEC014-1) with an *R279H p63* mutation (Fig 3), showed strong expression of MUC1, but a total absence of K12 staining (i.e., no corneal cells in the central cornea), thus indicating extensive conjunctivalization and confirming a complete loss of stem cells from the limbus. Defective limbal function produced corneal ulceration and subsequent corneal neovascularization and/or scarring in 56.5% (13/23) of cases (Fig 1A–C, G–I). The LSCD resulted in a progressive keratopathy with a dense vascularized corneal pannus, leading to visual impairment (Fig 2A), which was associated with advancing age (Fig 2B).

Table 1. Ocular Phenotype in Ectrodactyly-Ectodermal

Family	Mutation	Age*	Best-Corrected Visual Acuity†	Lacrimal Defects	Meibomian Glands	Lashes	Conjunctival scarring‡
EEC001	p.R304Q	30	0.8	+	Absent	T, M	
EEC002-1	p.R304Q	34	0.8	+	Absent	T	
EEC002-2	p.R304Q	10	1.0	+	Absent		
EEC003-1	p.R279H	33	0.5	+	Absent	T	IIc
EEC003-2	p.R279H	53	0	+	Absent	T	IId; IIIa
EEC003-3	p.R279H	9	1.0	+	Absent		
EEC004	p.R304Q	3	1.0	+	Absent		IIb
EEC005	p.R304Q	33	0.8	+	Absent	T	IIb
EEC006	p.R279S	9	1.0	+	Absent		
EEC007	p.R311G	44	1.0	+	Absent		IIIb
EEC008	p.S272N	39	0.8	+	+/−	T, D	IIc; IIIb
EEC009	p.R280C	27	1.0	+	Absent		
EEC010-1	p.H208R	39	0.33	+	Absent	T	
EEC010-2	p.H208R	60	0.17		Absent	T	I
EEC011	p.R279C	9	1.25	+	Absent		
EEC012	p.R304Q	18	0.17	+	Absent		IIIa; IIb
EEC013	p.S272T	22	0.8	+	Absent		
EEC014-1	p.R279H	48	0	+	Absent		IIIc; IIc-d
EEC014-2	p.R279H	29	1.0	+	Absent		
EEC015§	p.R304W	2	‡	+	Absent		
EEC016§	p.R204Q	2	‡	+	Absent		
EEC017	p.S272T	36	0.8		Absent		
EEC018	p.R279H	18	1.0	+	Absent		

+ = positive; − = negative; +/− = asymmetrical; A = aqueous deficiency; D = distichiasis; L = lipid defect and evaporative dry eye; LSCD = limbal stem cell deficiency; M = madarosis; NV = neovascularization; T = trichiasis.
*Age at examination (yrs).
†Best-corrected Snellen binocular vision (decimal fraction).
‡Conjunctival scarring: I. Subconjunctival scarring and fibrosis; II. Vertical disease: a. 0–25%, b. 25–50%, c. 50–75%, d. 75–100%; III. Horizontal disease:
§Owing to age, unable to assess best-corrected visual acuity.

There was, however, no apparent relationship of limbal stem cell failure with severity of EEC syndrome, as classified by EEC syndrome score, or the underlying molecular defect in *p63*.

When considering the prevalence of LSCD in EEC syndrome a number of issues should be considered, particularly study recruitment and ascertainment bias. All patient societies use the established EEC clinical phenotype as their diagnostic criteria and all patients were diagnosed by clinical geneticists. The UK Ectodermal Dysplasia Society was formed in 1997; the Italian societies were formed in 2009 owing to patient interest generated by this study. The UK patients were contacted in 2007 and there were 22 patients with EEC who were members of the society. Of the potential cohort, 50% (11/22) responded and attended the study. As for the Italian patients, 14 patients/families from different regions contacted The Veneto Eye Bank Foundation with the help of the 2 Italian associations. Approximately 86% (12/14) responded and attended the study. All the EEC patients that responded met the diagnostic criteria of the condition, and patients ranged widely in age. The study presented was not designed to be an epidemiologic assessment of LSCD in EEC syndrome; in this study, there could be a confounding ascertainment bias. The data from the cohort, however, strongly support the statement that the major cause of visual morbidity in EEC syndrome is LSCD.

Ocular Pathology in the EEC Syndrome

Characterization of the fibrovascular corneal pannus removed from a patient with EEC syndrome (EEC014-1) undergoing ocular surface reconstruction confirmed the presence of corneal conjunctivalization and LSCD (Fig 4). The phenotype of the pannus was conjunctival, with negative cornea-specific K12 staining (Fig 4D) and strong K19 (Fig 4B), MUC1 (Fig 4C), and MUC4 (Fig 4E) expression, thus confirming that the corneal epithelium was replaced by conjunctival overgrowth (conjunctivalization). Cells positive for CD45, a marker expressed in vascularized and inflamed cornea, were also observed (Fig 4F). Basal and nuclear expression of *p63* isoforms, including $\Delta Np63\alpha$, was observed (Fig 4A). The epithelium did not show a normal, well-organized, uniform, and multilayered stratification, but was reduced to just a single cell layer. Similarly, the histologic analysis of central corneal button obtained at the time of PK in individual EEC010-1 showed that the corneal epithelium was markedly atrophic and attenuated with destruction of Bowman's layer and anterior stromal fibrosis (Fig 5).

Outcome of PK in the EEC Syndrome

In this cohort 2 patients underwent PK in the 1990s (EEC003-1 and EEC010-1). Patient EEC003 had a complicated postoperative course with recurrent herpes simplex virus keratitis and graft failure. Patient EEC010-1 (Fig 6) underwent a right PK in 1994 (at age 36) and a left PK in 1998 (at age 40) because his vision was severely compromised (0.04; decimal fraction) owing to corneal scarring and neovascularization. Postoperatively his visual acuities fluctuated between 0.5 and 0.32 (decimal fraction) because of recurrent epithelial breakdown that was managed with a long-term, monthly bandage contact lens until his death at age 43 from syringocystadenocarcinoma papilliferum.

Discussion

The EEC syndrome is caused by mutations in the *p63* gene, an important transcription factor during embryogenesis and for stem cell differentiation in stratified epithelia. Familial cases show an autosomal-dominant inheritance with marked intrafamilial and interfamilial variability. However, the ma-

Dysplasia-Clefting (EEC) Syndrome Cohort

Tear Film Anomaly	LSCD	Corneal Ulceration	Corneal NVs	Corneal Scarring	Cataract	EEC Score
L					+	13/18
L	+	+	+	+		17/18
L						7/18
L, A	+	+	+	+		12/18
L, A	+	+	+	+		9/18
L, A						16/18
L		+				16/18
L, A	+	+	+	+		11/18
L						15/18
L, A	+	+	+			14/18
L	+	+		+		16/18
L, A						11/18
L, A	+	+	+	+		14/18
L	+	+	+	+	+	5/18
L						15/18
L, A	+	+	+	+		14/18
L, A	+/−	+		+/−		10/18
L, A	+	+	+	+		5/18
L, A	+/−					8/18
L						12/18
L						9/18
L, A	+/−			+/−		7/18
L, A	+/−					12/18

a. 0–25%, b. 25–50%, c. 50–75%, d. 75–100%; IV. Ankyloblepharon, frozen globe, totally keratinized.

jority of cases are sporadic, resulting from de novo mutations arising during the first stage of embryonic development. The *p63* gene is a transcription factor belonging to the same protein family as *p53* and *p73*.[25] The *p63* gene contains 15 exons and encodes 2 different classes of proteins: The transcriptionally active full-length *TAp63* (the TA isotypes), and the amino-terminally truncated dominant-negative Δ*Np63* (the ΔN isotypes).[24] Differential promoter usage and alternative splicing generates 6 *p63* isoforms[26] that all contain a DNA-binding and isomerization domain. The α isotypes encode a sterile alpha motif domain, a key region with a putative role in protein–protein interactions during embryologic development.[27]

Mutations in the *p63* gene can cause ≥6 different syndromes: EEC syndrome (MIM#604292), Rapp–Hodgkin syndrome (MIM#129400), Hay–Wells syndrome or ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (MIM#106260), limb mammary syndrome (MIM#603543), acro-dermato-ungual-lacrimal-tooth syndrome (MIM#103285), and split-hand and foot malformation type 4 (MIM#605289).[23] Mutations in *p63* account for 98% of patients with typical EEC features; the majority of mutations are heterozygous missense mutations located in the DNA-binding domain.[14] Only 2 *p63* mutations have been reported outside the DNA-binding domain: One insertion (c.1572insA) and one point mutation (p.L563P), both in the sterile alpha motif domain.[4,28] To date, approximately 40 different pathogenic *p63* mutations have been identified in EEC syndrome (for review see Clements et al[29]).

In our study, 2 unrelated patients (EEC013 and EEC017) of Italian descent had a novel *p63* mutation (p.S272T); all other detected mutations have previously been reported.[29] The arginine codons at 304 and 279 were mutational hotspots and an arginine residue was mutated in almost three quarters of the EEC cases. This reflects the literature; nearly 90% of *p63* mutations in EEC syndrome involve 5 arginine residues in the DNA-binding domain: p.R204, p.R227, p.R279, p.R280, and p.R304.[24] This mutational spectrum is relevant to the design of strategies to detect mutations in the *p63* gene in EEC syndrome.[30] The arginine residues (Arg 204, 227, 279, and 280) represent C>T transitions at the high mutabile 5-methylcytosine at CpG sites[31] and correspond exactly to somatic p53 mutations hotspots in human cancers,[32] thus suggesting that these sites are foci for DNA damage and that this damage is poorly repaired.[33] The high frequency (>60%) of de novo mutations found in pa-

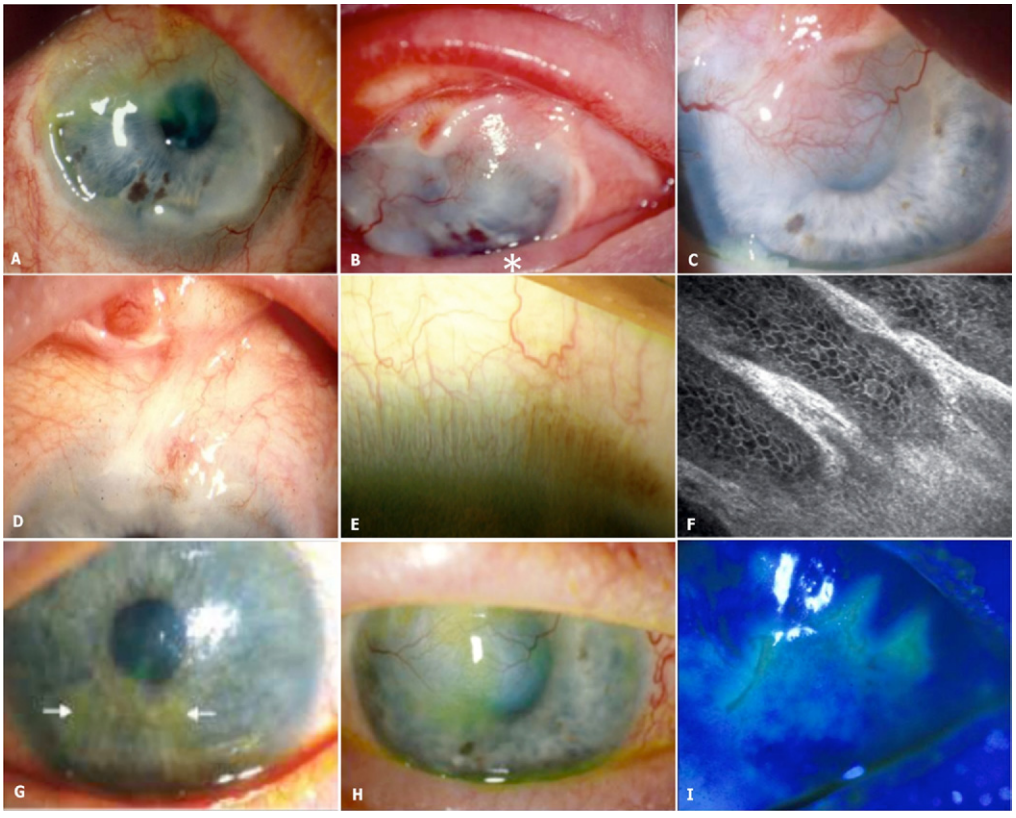

Figure 1. Ocular phenotype in patients with ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome. **A–D,** Opaque corneas with radial new vessels and ocular surface disease **B**, Absence of meibomian glands on the lid margin (*). The loss of the palisades of Vogt (i.e., the niche of corneal stem cells) has been evaluated by slit-lamp (**E**, a small portion of a normal limbus: Note the ridges and pigment characteristic of the Palisades of Vogt); in vivo laser scanning confocal microscopy examinations (**F**, normal corneal limbus demonstrating the palisades of Vogt, often degenerated or totally absent in EEC patients); and by fluorescein staining, an orange stain that is applied to the cornea to reveal corneal lesions, because the stain adheres to areas where the surface layer of the cornea (the epithelium) is missing and where the underlying layer (corneal collagen or stroma) has been exposed. **G**, **H**, Corneal epithelial defect (arrows) stained with 2% fluorescein in a young patient **I**, Cornea viewed with cobalt blue filter producing fluorescence of epithelial defect and enhancing visualization.

tients with EEC syndrome supports this mechanism. Mutation of these arginine residues is highly detrimental to DNA binding and transactivation activity.[24] All patients with the EEC syndrome involved in our study had ocular features irrespective of the severity of their systemic manifestations (EEC score) or specific *p63* mutation. Of note, all patients with EEC had meibomian gland defects. Complete absence of the meibomian glands is very rare in the population, but it has been reported in patients with EEC syndrome and anhidrotic ectodermal dysplasia.[5,11,12,34,35] Absence of the meibomian glands and deficiency of the lipid layer was seen in all EEC patients in this study and as such indicates that an absence of the meibomian glands supersedes lacrimal duct anomalies as the commonest ocular defect.[1–3]

This study confirms that tear film instability and a rapid tear breakup time, resulting in an evaporative dry eye, is the primary tear abnormality in EEC syndrome, thus supporting other authors' experiences.[5,11,35] Reduced lacrimal gland secretion in the EEC syndrome results in aqueous tear deficiency, as reported previously.[5,9,33,36] There have been reports of individuals with EEC syndrome with normal aqueous tear secretion,[12,13,36–38] and aqueous secretion was normal in approximately 40% of cases in this cohort. The mucinous layer may also be deficient, with reduced numbers of conjunctival goblet cells,[11] compounding tear abnormalities resulting from meibomian gland absence and reduced lacrimal secretion leading to an exacerbation of the evaporative dry eye state.

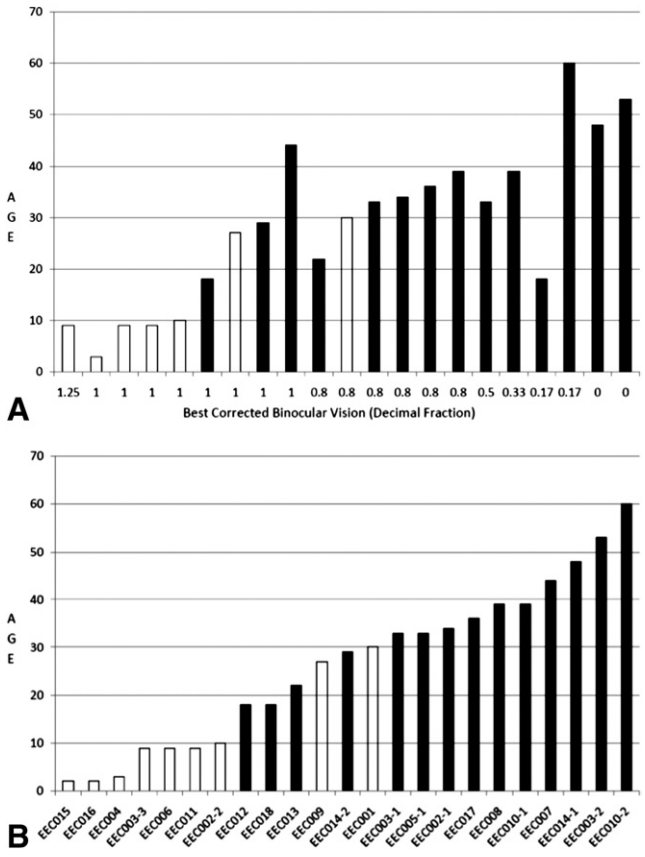

Figure 2. **A**, Visual acuity, patient age, and limbal stem cell deficiency (LSCD) in ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome. The EEC patients with LSCD are shown with black bars. Note poor visual acuity associated with presence of LSCD. **B**, Increasing LSCD with age in EEC syndrome. The EEC patients with LSCD are shown with black bars. The x axis in panel B is the patient identification number from Table 1.

Lacrimal system defects have been reported in 59% to 100% of EEC syndrome cases[1,2] and in 87% in the most complete UK-based study.[3] Lacrimal drainage system defects were the second commonest ocular anomaly and were present in 91.3% of EEC patients in this cohort. The lacrimal drainage system abnormalities seen in this study reflect those reported in the literature and include absence, stenosis or occlusion of the puncta and/or canaliculi, lacrimal fistula, nasolacrimal duct stenosis, and obstruction including complete absence of the membranous and bony nasolacrimal duct.[8] These abnormalities can lead to epiphora from birth, bilateral dacyrocystitis, lacrimal abscess, and mucocele.[7,8]

More important, our study confirms that the major cause of visual morbidity in EEC syndrome is limbal stem cell failure, which causes a progressive keratopathy resulting in a dense, vascularized corneal pannus, leading to progressive visual impairment. Defective limbal function produced corneal ulceration and subsequent corneal neovascularization and/or scarring with progressive visual impairment. Our hypothesis is that LSCD results from mutations in *p63*.

The *p63* gene is essential for regenerative proliferation in epithelial development, distinguishes human keratinocyte stem cells from their transient amplifying cell progeny, is expressed by the basal cells of the limbal epithelium (but not by transient amplifying cells covering the corneal surface), and is abundantly expressed by epidermal and limbal holoclones but is undetectable in paraclones.[39,40] In the human corneal epithelia, $\Delta Np63\alpha$ is the major *p63* isoform. Limbal–corneal keratinocytes express not only $\Delta Np63\alpha$ but also the $\Delta Np63\beta$ and $\Delta Np63\gamma$ isoforms. However, although expression of $\Delta Np63\alpha$ is restricted to the limbal stem cell compartment, the expression of $\Delta Np63\beta$ and $\Delta Np63\gamma$ correlates with limbal cell migration, corneal wound healing, and corneal differentiation.[40,41] Therefore, *p63* plays a key role in corneal epithelia and explains why mutations might lead to defective limbal stem cell function and progressive keratopathy. Interestingly, histologic examination of the central cornea after PK in 1 individual with the EEC syndrome (EEC010-1; Fig 6) demonstrated marked corneal epithelial attenuation and atrophy, which is in keeping with previous reports of corneal pathology in EEC syndrome.[11,12]

The identification of LSCD as the major cause of visual morbidity has important consequences. The management of the corneal disease associated with EEC syndrome is challenging because treatments are not aimed at the underlying condition and so are mainly supportive with variable clinical effect.[5,7,12] The combination of ocular surface disease, tear film abnormalities, corneal neovascularization, poor epithelial healing, and ectodermal dysplasia make patients with EEC syndrome high-risk candidates for corneal surgery.[5,12,38] Mader and Stulting[6] reported the outcome of PK in 2 related patients with EEC syndrome who underwent surgery after a spontaneous corneal perforation. Postopera-

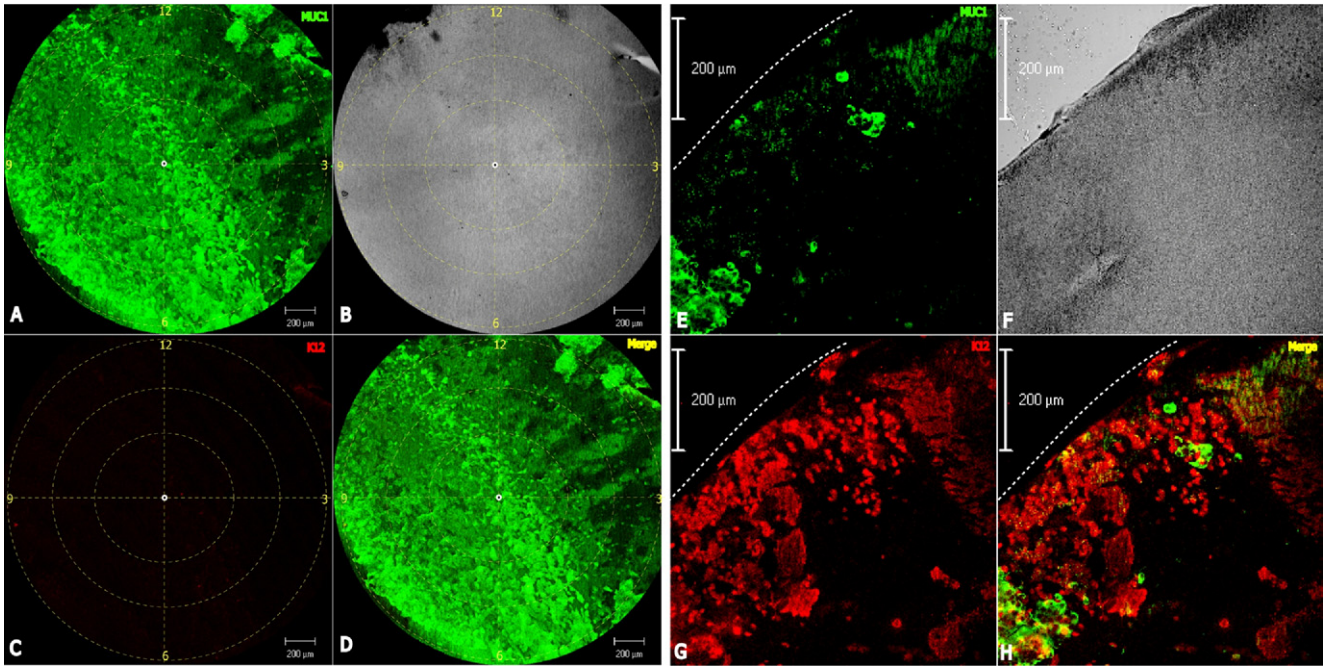

Figure 3. Impression cytology in ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome. Specimens were stained with antibodies against keratin 12 (K12, a typical corneal marker) and mucin 1 (MUC1, more abundant in the conjunctiva) and signals quantified through quantification of fluorescence immunohistochemistry analysis. Example is shown for one patient (EEC014-1 from Table 1). For each panel, staining is shown counter-clockwise: MUC1 (green, **A**), confocal microscope grid (grey, **B**), K12 (red, **C**), and merge (**D**). Immunostaining with MUC1 and K12 showed a completely conjunctivalized cornea. No overlapping signals were seen between K12 (red) and MUC1 (green). (**E**–**H**) Details of 1 healthy area show that corneal cells positive for K12 (**G**) were rare and only seen in 1 peripheral part of 1 impression cytology specimen, suggesting a potential residual healthy area that could be used in the future to isolate corneal epithelial stem cells. Scale bar = 200 μm.

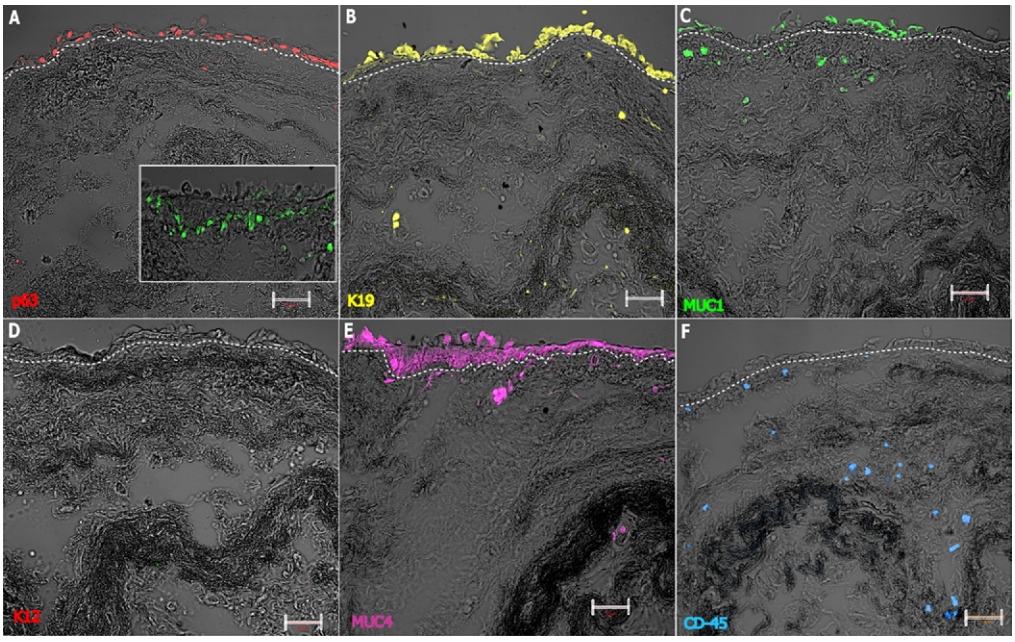

Figure 4. Immunohistochemistry of fibrovascular pannus in ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome. Characterization of the corneal pannus removed from a patient affected by EEC syndrome (EEC014-1 patient in Table 1). Presence of conjunctival markers and lack of keratin 12 (K12) confirmed the clinical diagnosis, namely, corneal conjunctivalization and limbal stem cell deficiency. Immunofluorescent staining is shown for *p63* (**A**, red), Δ*Np63α* (in box of panel **A**, green), K19 (**B**, yellow), mucin (MUC)1 (**C**, green), K12 (**D**, red), MUC4 (**E**, purple), and CD-45 (**F**, blue). Scale bar = 50 μm.

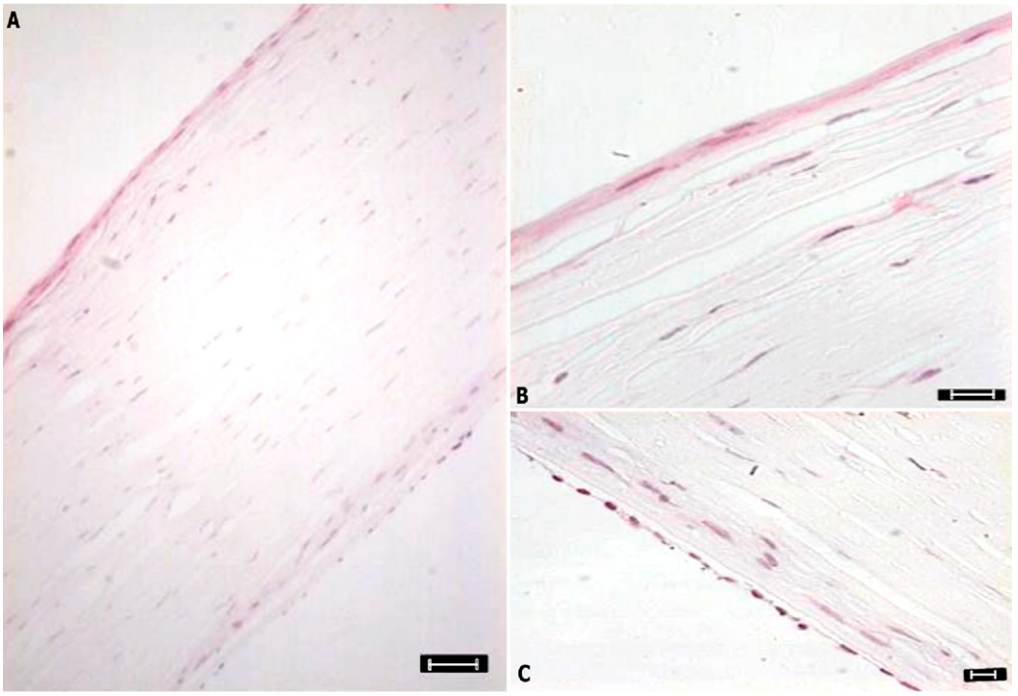

Figure 5. Histopathology of corneal button after penetrating keratoplasty in ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome. Hematoxylin-stained cross sections of a cornea affected by EEC syndrome (EEC010-1 patient in Table 1). Specimen consisted of an epithelium with reduced degrees of epithelial stratification. Corneal epithelium was markedly atrophic and attenuated with destruction of Bowman's layer and anterior fibrosis (**A**, **B**), resulting in severe tissue hypoplasia. There was a patchy increase in keratocyte density anterior to Descemet's membrane, which was intact, and the endothelium was confluent (**C**). Scale bars are 93 μm (**A**) and 23 μm (**B** and **C**).

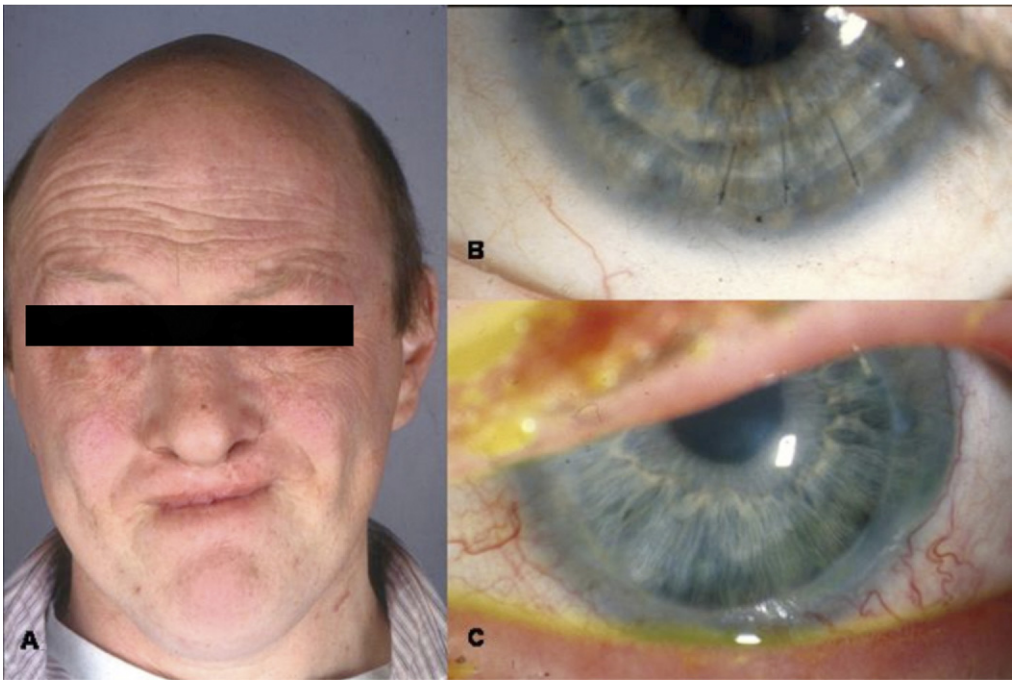

Figure 6. Clinical and ocular features of ectrodactyly-ectodermal dysplasia-clefting (EEC) patient 010-1. Facial appearance note cleft lip repair, skin appearance, scalp alopecia, short philtrum, and blepharospasm with photophobia (**A**). Appearance of right penetrating keratoplasty (PK) 6 years postoperatively (**B**). Appearance of left PK 3 years postoperatively, giving a best-corrected visual acuity of 0.32 (**C**).

tively, one developed microbial keratitis and attained a visual acuity of 20/100 at 10 months postoperatively; the other had recurrent epithelial erosions postoperatively, and after 22 months of follow-up the donor cornea had developed peripheral scarring and neovascularization with a visual acuity of 20/60.[6] Baum et al[12] described a child with EEC syndrome who, at the age of 3, suffered from severe photophobia and had a vascularized scar and ulceration in the right eye. Penetrating PK was performed when the child was 5 years old, but 1 month postoperatively only 1 to 2 mm of the donor button had re-epithelialized; corneal graft subsequently opacified and failed. Our study highlights the correlation of LSCD with EEC syndrome owing to mutations in the *p63* gene, so this is outcome is not unexpected; the donor's healthy epithelium is soon replaced by the patient's altered (owing to mutations in *p63*) corneal epithelial cells. A keratolimbal allograft offers the potential to treat the underlying limbal stem cell failure, but reports of this approach in EEC syndrome are limited with variable success.[42,43] More recently, a patient with ectodermal dysplasia was grafted with allogenic cultured limbal stem cells obtained from cadaveric human corneas, but the treatment was similarly unsuccessful.[44] More important, both keratolimbal allograft and allogenic limbal stem cell grafting require life-long immunosuppression and its potential side effects. For these reasons, and based on the results obtained in this study, our view is that only gene therapy–based approaches might lead to some benefit for the ocular pathology affecting EEC patients. The EEC syndrome results from heterozygous dominant-negative mutations in the *p63* gene. Therefore, therapeutic strategies based on allele-specific gene silencing through RNA interference in harvested limbal stem cells could inhibit specifically the expression of disease-associated alleles without suppressing the expression of the corresponding wild-type alleles.[45,46]

To our knowledge, this is the first study correlating the ocular features of EEC patients with *p63* mutations and not limited to just genetic[4,23,28] or clinical characterization of patients.[8] As clearly demonstrated by clinical data and failure of limbal stem cells to generate a fully stratified corneal epithelium in our study, LSCD results from mutations within the *p63* gene. Even if molecular mechanisms leading to LSCD remain to be elucidated, our study helps to identify potential therapeutic strategies for the ocular disorders affecting EEC patients.

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