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CLINICAL INVESTIGATION

In vivo histology and p.L132V mutation in *KRT12* gene in Japanese patients with Meesmann corneal dystrophy

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Abstract

Purpose To report genetic mutational analysis and *in vivo* histology of Meesmann corneal dystrophy.

Study design Prospective, case control study.

Methods Six patients from three independent families with clinically diagnosed Meesmann corneal dystrophy were enrolled in this study. Slit-lamp biomicroscopy with fluorescein vital staining, anterior segment optical coherence tomography (AS-OCT), and *in vivo* laser confocal microscopy (IVCM) were performed on selected patients. Mutational screening for the keratin genes *KRT3* and *KRT12* was performed in all six patients and selected unaffected family members.

Results Slit-lamp biomicroscopy revealed numerous intraepithelial microcysts in all affected individuals. AS-OCT revealed hyperreflectivity and high corneal epithelial layer thickness (mean, 64.8μm) in all individuals tested (3/3). By using IVCM, multiple epithelial microcysts and hyperreflective materials (6/6), subepithelial nerve abnormalities (6/6), tiny punctate hyperreflective material (6/6), and needle-like hyperreflective materials (4/6) were observed in the corneal stromal layer. A heterozygous genetic mutation in the *KRT12* gene (c.394 C>G, p.L132V) was identified in all six patients. No pathological mutation was observed in the *KRT3* gene.

Conclusion We identified a heterozygous genetic mutation (c.394 C>G, p.L132V) in the *KRT12* gene in six Japanese patients with inherited Meesmann corneal dystrophy. This is the first study to confirm this genetic mutation in Japanese Meesmann corneal dystrophy patients. This mutation has been independently reported in an American Meesmann corneal dystrophy patient, confirming its pathogenicity. AS-OCT and IVCM proved to be useful tools for observing corneal epithelial layer pathology in this dystrophy. Furthermore, IVCM reveals corneal stromal layer pathological changes not previously reported in this dystrophy.

Keywords Meesmann corneal dystrophy · Anterior segment optical coherence tomography · *In vivo* confocal microscopy · Genetic mutation analysis · Corneal epithelium

Introduction

Meesmann corneal dystrophy (OMIM 122100) is a rare dominantly inherited epithelial corneal dystrophy [1]. This dystrophy can be clinically diagnosed on slit-lamp examination, which reveals bilateral multiple microcysts and fine punctate opacities in the corneal epithelium. Although the dystrophy is generally mild and asymptomatic, some patients present with excess lacrimation, foreign body sensation, photophobia, and impaired vision. Patients are sometimes misdiagnosed with dry eye syndrome due to the clinical manifestations. This dystrophy is usually considered to not affect the corneal stromal layer or endothelial cell layer [1].

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Meesmann corneal dystrophy is known to be associated with mutations in cornea-specific keratin 3 (*KRT3*) and keratin 12 (*KRT12*) genes. These genes encode keratins, the intermediate filament proteins primarily responsible for imparting mechanical strength and structural support to corneal epithelial cells [2, 3]. To date, 4 mutations in the *KRT3* gene and 24 mutations in the *KRT12* gene have been reported in this dystrophy [2–22]. Among the 28 mutations of this dystrophy, 8 have been identified in the *KRT12* gene in Japanese patients [3, 8, 17, 21], but none in the *KRT3* gene.

Recent advances in imaging device technology in the corneal research and clinical practice enable *in vivo* layer-by-layer analysis of the entire cornea. The technology includes *in vivo* laser confocal microscopy (IVCM) and anterior segment optical coherence tomography (AS-OCT) [15, 21, 23–27]. Both IVCM and AS-OCT allow non-invasive, real-time investigations of corneal microstructures and both are useful for clinical diagnosis and follow up of corneal diseases.

In this study we performed mutational analysis of Japanese patients with inherited Meesmann corneal dystrophy and of their families that revealed a missense mutation in the *KRT12* gene (c.394 C>G, p.L132V) which is novel in Japanese patients but has independently been reported in an American Meesmann corneal dystrophy patient. We also used both AS-OCT and IVCM and investigated *in vivo* microstructural changes of the entire cornea in eyes afflicted with this dystrophy.

Materials and methods

The prospective research plan for this study was approved by the Human Genome/Gene Analysis Research Ethics Committee of Kanazawa University (Approval number: 168), and human patient sample collection strictly followed the ARVO statement on human subjects and the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all participants before enrollment into this study.

A total of 6 Meesmann corneal dystrophy patients and 4 unaffected family members were enrolled in this study. The 10 subjects were members of three independent families (Fig. 1). Subjects were first examined using slit-lamp biomicroscopy with or without fluorescein vital staining and photographed with a slit-lamp camera. AS-OCT and IVCM were performed upon written informed consent followed by blood sample collection and testing for genetic mutational analysis using polymerase chain reaction (PCR).

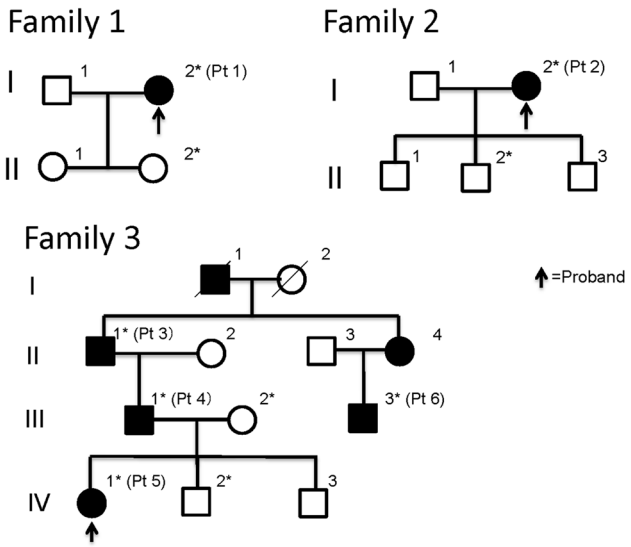

Fig. 1 Pedigree of Family 1 (Top left), 2 (Top right) and 3 (second row). The arrow indicates the proband (I-2 in family 1 and 2, IV-1 in family 3) and the asterisk indicates the genetically tested individual

Anterior segment optical coherence tomography (AS-OCT)

Selected patients were examined using AS-OCT (RTVue-100® Optovue Inc.) with a corneal adaptor module. The system captures 26000 A-scans per second and has a laser diode of 840nm and an axial resolution of 5µm. All images were captured by a trained operator. Images were evaluated with special attention to changes in the degree of light reflection in the corneal epithelium. Total corneal thickness and corneal epithelial thickness maps were generated for AS-OCT images over the 6-mm-diameter corneal area and analyzed quantitatively.

In vivo laser confocal microscopy (IVCM)

After applying a large drop of contact gel (Comfort Gel ophthalmic ointment®, Bausch & Lomb) on the front surface of the microscope lens and ensuring no air bubbles had formed, a Tomo-cap® (Heidelberg Engineering GmbH) was mounted on the holder to cover the microscope lens. The cap was fixed on the center of the cornea and the cornea was examined layer by layer by the Heidelberg Retina Tomograph 2 Rostock Cornea Module (HRT2-RCM) with a 60× water-immersion objective lens (Olympus Europa GmbH). The HRT2-RCM utilizes a 670-nm diode laser as the light source with a 400×400µm area of observation.

Genetic mutational analysis

Mutational screening for the *KRT3* and *KRT12* gene was performed in all 6 Meesmann corneal dystrophy patients from three independent families as well as 4 unaffected family members. After obtaining informed consent from all subjects including 102 unrelated normal volunteers, genomic DNA was extracted from the peripheral blood using a DNA extraction kit (QIAmp Blood Maxi Kit). The exonic regions of the *KRT3* and *KRT12* were amplified by polymerase chain reaction (PCR) using previously published forward and reverse primers [3, 9, 15]. PCR products were purified with the High Pure PCR production Kit (Roch Diagnostics), subjected to a dideoxy chain termination reaction using BigDye Termination v1.1 Cycle Sequencing Kit (Life Technologies), and run bidirectionally on an ABI PRISM 3130 Genetic Analyzer (Life Technologies). The nucleotide and amino acid sequences of *KRT3* and *KRT12* were blasted against the NCBI reference sequences for *KRT3* (Genbank Accession:NM_057088.2) and *KRT12* (Genbank Accession:NM_000223.3). Identified sequence variants were annotated according to the Human Genome Variation Society nomenclature guidelines. The Single Nucleotide Polymorphism Database (dbSNP) was used to identify the minor allele frequencies of obtained variants (https://www.ncbi.nlm.nih.gov/snp).

Results

Table 1 summarizes demographic, clinical, and slit-lamp biomicroscopic results for the 6 Meesmann corneal dystrophy patients enrolled in this study.

Slit-lamp biomicroscopy

In the probands and all patients, Slit-lamp biomicroscopy revealed numerous intraepithelial microcysts OU (Figure 2, Table 1). A clear zone with no epithelial lesions was observed in one young patient only (8 years old, family 3, IV-1) (Fig. 2 c, d; Table 1). Whitish corneal opacities were observed in the rest of the patients. On the other hand, slit-lamp biomicroscopy revealed no corneal lesions in any of the unaffected family members.

Anterior segment optical coherence tomography (AS-OCT)

AS-OCT revealed hyperreflectivity in the epithelial layer and in Bowman's layer in all tested patients (Fig. 3, Table 2). No other abnormalities were detected in the remaining corneal layers. The mean corneal thickness of each eye is reported based on the AS-OCT system's software output which produced a total corneal thickness mean as well as a corneal epithelial layer thickness mean over 6-mm-diameter corneal area. As shown in Figure 4, central epithelial thickness was the highest at 75μm in patient 3, and average corneal epithelial thickness of all three patients tested was 64.8μm (OD/OS 61/75μm in patient 3, 66/69μm in patient 4, 57/61μm in patient 5), higher than previous reports demonstrating normal epithelial thickness as 53.0±2.7μm [24].

In vivo laser confocal microscopy (IVCM)

Using IVCM, we observed in all patients multiple microcysts and hyperreflective material in the corneal epithelium (6/6). Surprisingly, tortuous and hyporeflective subepithelial

Table 1 Clinical data and genetic findings of six patients with Meesmann corneal dystrophy

Patient	Family (family number, see Figure 1)	Age	Sex	Symptom	BCVA (OD/OS)	Slit lamp examination		Gene mutation (amino acid change)	
						microcysts	clear zone	*KRT3*	*KRT12*
1	1 (I-2)	40	F	FBS, photophobia	1.0/0.6	+	-	none	c.394C→G (p.L132V)
2	2 (I-2)	62	F	FBS, photophobia	1.0/1.0	+	-	none	c.394C→G (p.L132V)
3	3 (II-1)	66	M	FBS, photophobia (episode of recurrent corneal erosion)	1.2/1.0	+	-	none	c.394C→G (p.L132V)
4	3 (III-1)	41	M	FBS, photophobia (episode of recurrent corneal erosion)	1.2/1.2	+	-	none	c.394C→G (p.L132V)
5	3 (IV-1)	8	F	FBS, photophobia (episode of recurrent corneal erosion)	1.2/1.2	+	+	none	c.394C→G (p.L132V)
6	3 (III-3)	43	M	FBS, photophobia (episode of recurrent corneal erosion)	1.2/1.2	+	-	none	c.394C→G (p.L132V)

F female, *M* male, *FBS* foreign body sensation, *BCVA* best corrected visual acuity, *OD* oculus dexter, *OS* oculus sinister

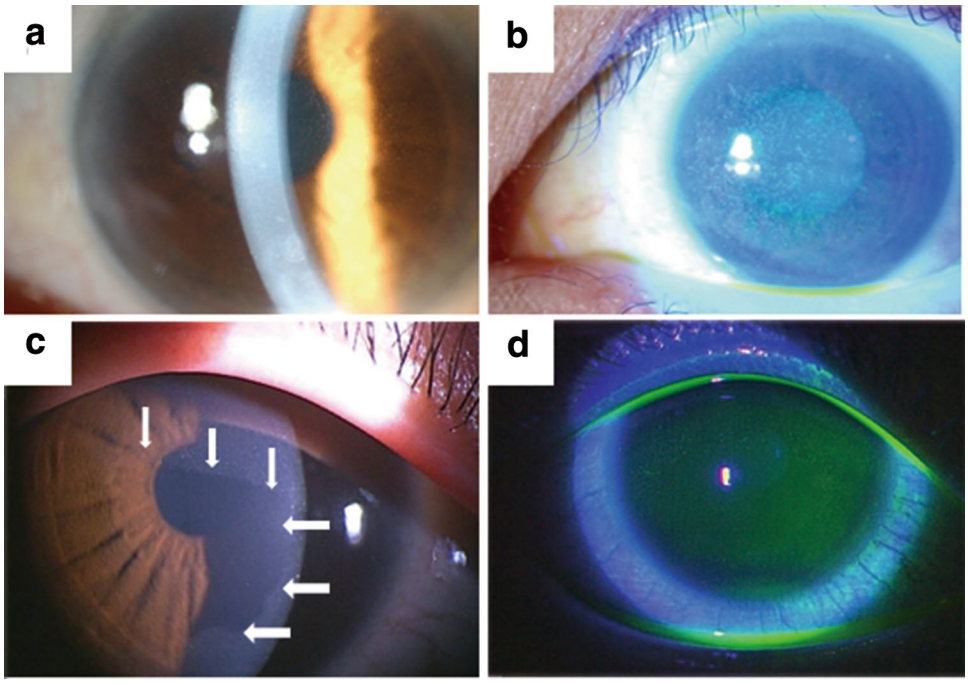

Fig. 2 Corneal slit-lamp biomicroscopy images of Meesmann corneal dystrophy patients. a. Slit-lamp biomicroscopy showed numerous intraepithelial microcysts (40 years of age: family 1, I-2). b. Slit-lamp biomicroscopy with fluorescein vital staining showed numerous diffuse superficial punctate keratitis-like opacities. c. A clear zone (arrows) was observed in one young patient only (8 years of age: family 3, IV-1). d. Slit-lamp biomicroscopy with fluorescein vital staining of the same young patient (family 3, IV-1) showed a clear zone without superficial punctate keratitis-like areas

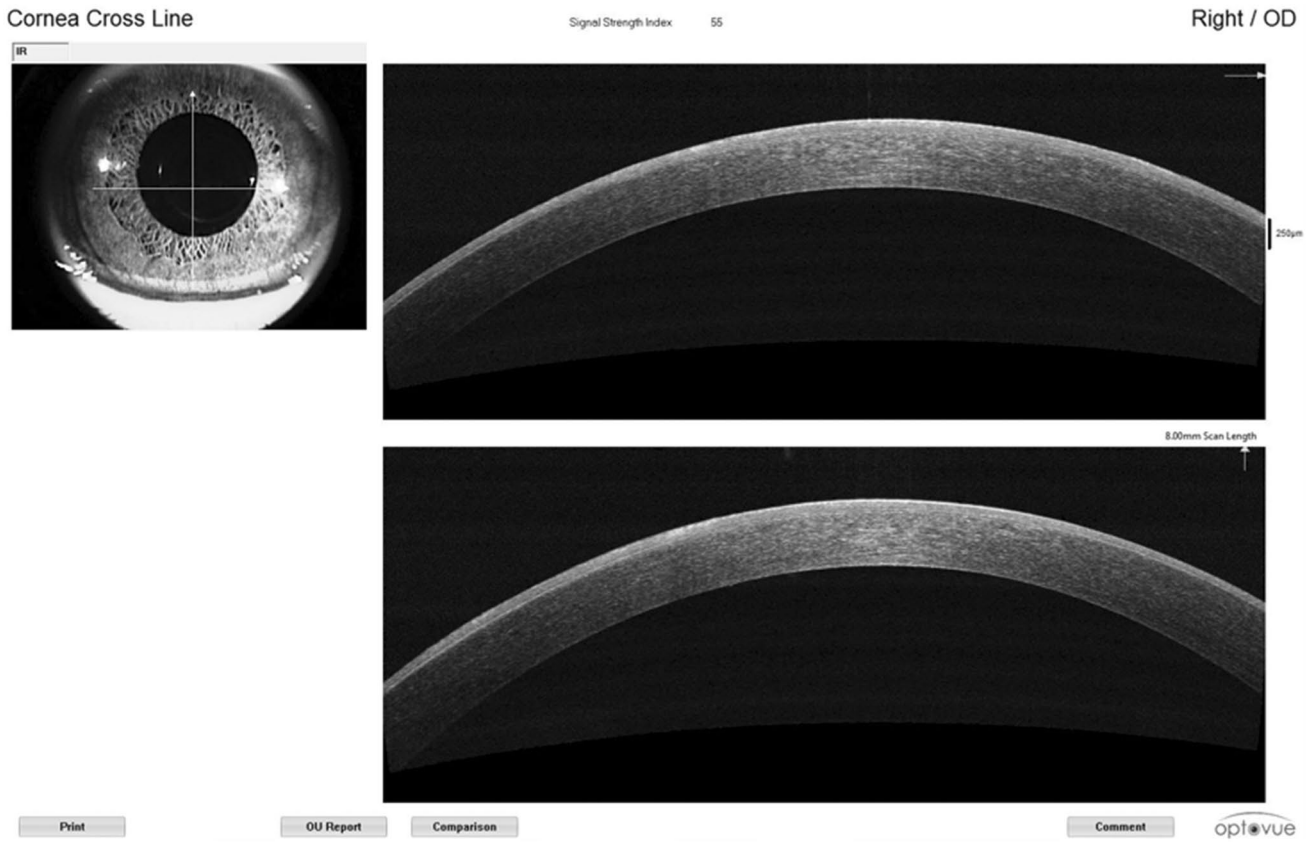

Fig. 3 Anterior segment optical coherence tomography (AS-OCT). AS-OCT revealed hyperreflectivity in the corneal epithelial layer and Bowman's layer in all patients tested. No other abnormalities could be detected in the remaining corneal layers

Table 2 AS-OCT and *in vivo* confocal microscopic findings of six patients with Meesmann corneal dystrophy

Patient	AS-OCT		IVCM			
	Hyperreflectivity in the epithelial layer	Epithelial layer thickening	Subepithelial nerve abnormalities	Tiny punctiform hyperreflective material in the stroma	Needle-like hyperreflective material in stroma	Bowman's layer atrophy
1	N/A	N/A	+	+	+	+
2	N/A	N/A	+	+	+	+
3	+	+	+	+	+	-
4	+	+	+	+	-	-
5	+	+	+	+	-	-
6	N/A	N/A	+	+	+	+

AS-OCT anterior segment optical coherence tomography, *IVCM* in vivo laser confocal microscopy, *N/A* not available

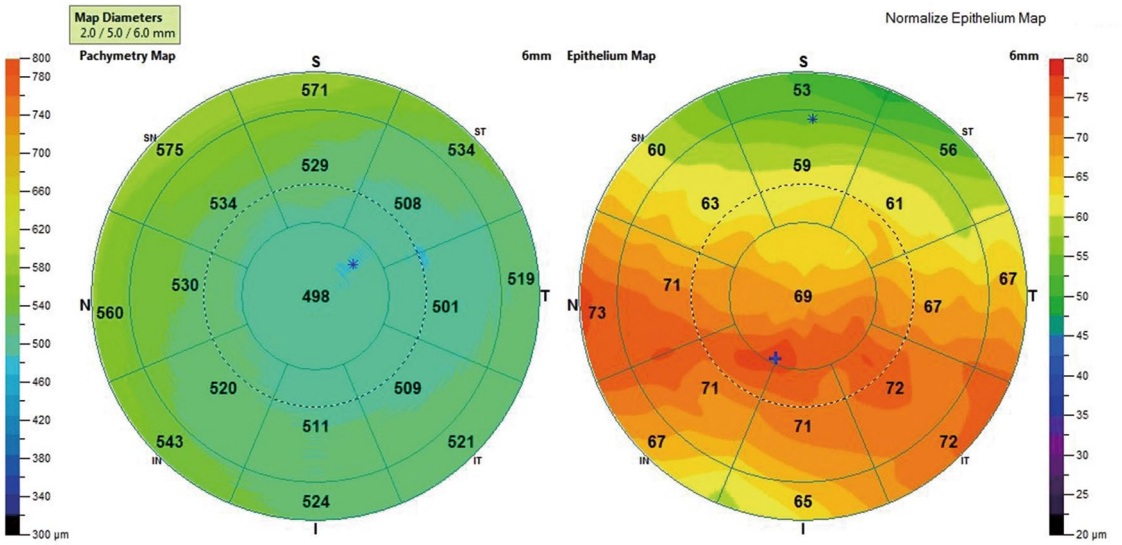

Fig. 4 Corneal epithelial thickness map generated from anterior segment optical coherence tomography (AS-OCT). Total corneal thickness map (left) and corneal epithelial thickness map (right) of patient 4. High corneal epithelium thickness was observed in all patients tested with a mean thickness of 64.8µm

nerve and tiny punctate hyperreflective material in the corneal stromal layer were also found in all patients (6/6). Furthermore, needle-like hyperreflective material was observed in the corneal stromal layer in 4 patients (Table 2, Fig. 5).

Genetic mutational analysis

Bidirectional sequencing of the *KRT12* gene revealed a heterozygous missense mutation from cytosine to guanine at nucleotide position c.394 (c.394C>G), predicting substitution of a valine for a leucine at codon 132 (p.L132V, CTT>GTT) in exon 1 of all patients (Fig. 6, Table 1). A heterozygous missense variation of p.D248N (c.742G>A, GAC>AAC) registered as a single nucleotide polymorphism (SNP) (rs150674571) was also detected in exon 12 of all patients. Neither the mutation nor the SNP was identified in the unaffected family members of the proband or in 204 control chromosomes. No other causative nucleotide alterations were found in the remaining coding exons of the *KRT12* gene. No mutation or variation in *KRT3* was detected in any of the patients or their unaffected family members.

Discussion

We performed a genetic mutational analysis of 6 Japanese patients with Meesmann corneal dystrophy from three independent families and found that all patients carry the same *KRT12* mutation (c.394C>G, p.L132V). This mutation is novel in Japanese patients and has been independently reported in an American Meesmann corneal dystrophy patient (Table 3) [12]. Considering that the three

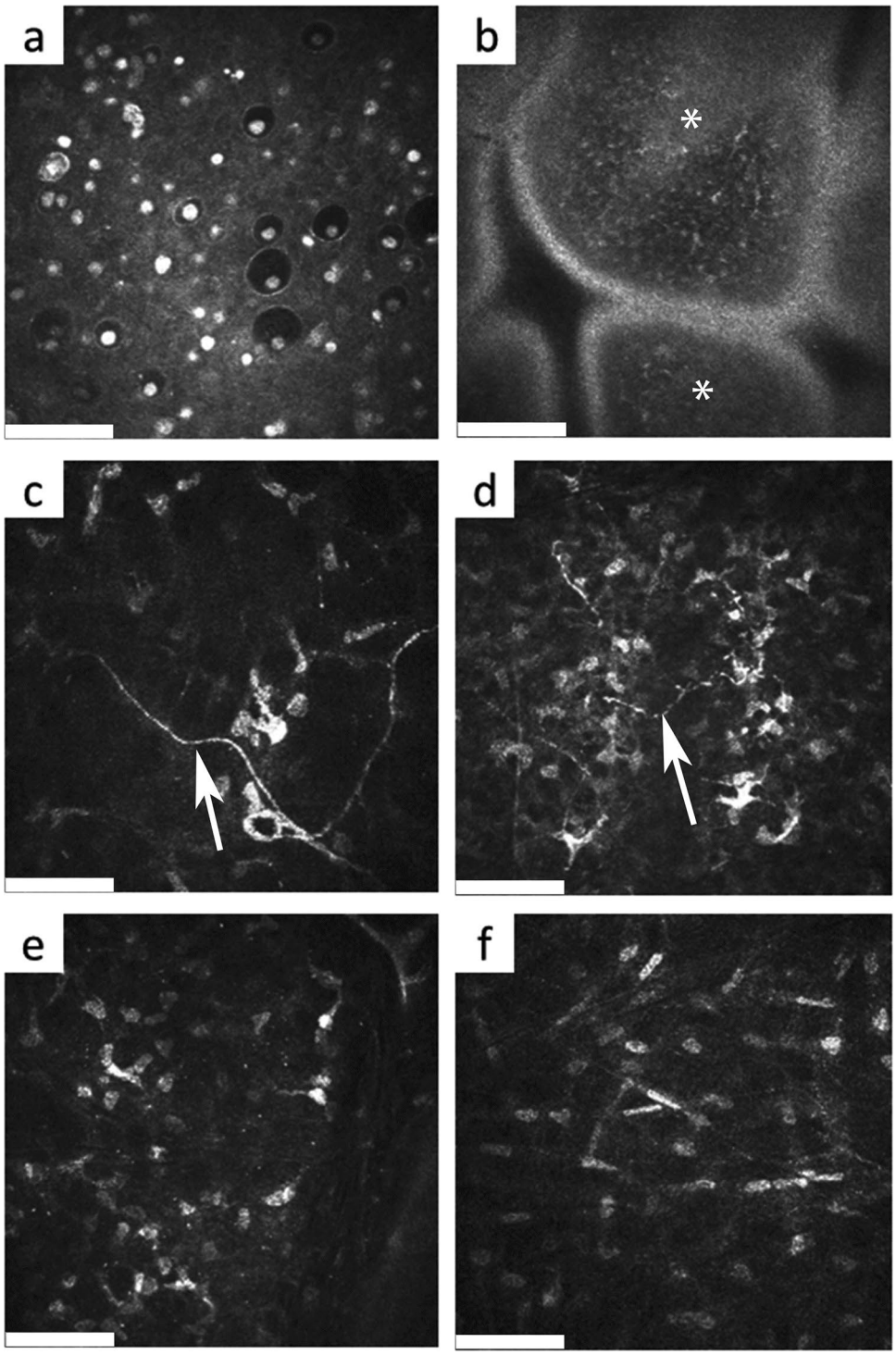

Fig. 5 Representative images of in vivo laser confocal microscopy (IVCM) in affected individuals of family 3 (white bar=100μm). a. Multiple microcysts and hyperreflective material were observed in the corneal epithelium. b. Atrophy of Bowman's layer was observed (asterisks). c., d. Tortuous and hyporeflective subepithelial nerve were observed (arrows). e. Tiny punctiform hyperreflective material in the corneal stromal layer were found. f. Needle-like hyperreflective material was observed in the corneal stromal layer

independent families carry the same *KRT12* mutation and live relatively near each other in Toyama prefecture in Japan, we surmise that they may have had a common ancestor. All other eight *KRT12* mutations within the helix initiation motif in Japan reported to date were from other districts.

Keratins are composed of 3 main parts: the central α-helical rod domain, divided into four subdomains (1A, 1B, 2A and 2B), and the 2 non-helical variable domains (V1 and V2) at each end [28]. All but one (27 bp insertion) of the causative mutations reported to date are missense substitutions that affect one terminal of the central α-helical rod domain in the *KRT3* and *KRT12* genes. Four mutations in the *KRT3* gene and 24 mutations in the *KRT12* gene have been previously reported (Table 3). Among the mutations

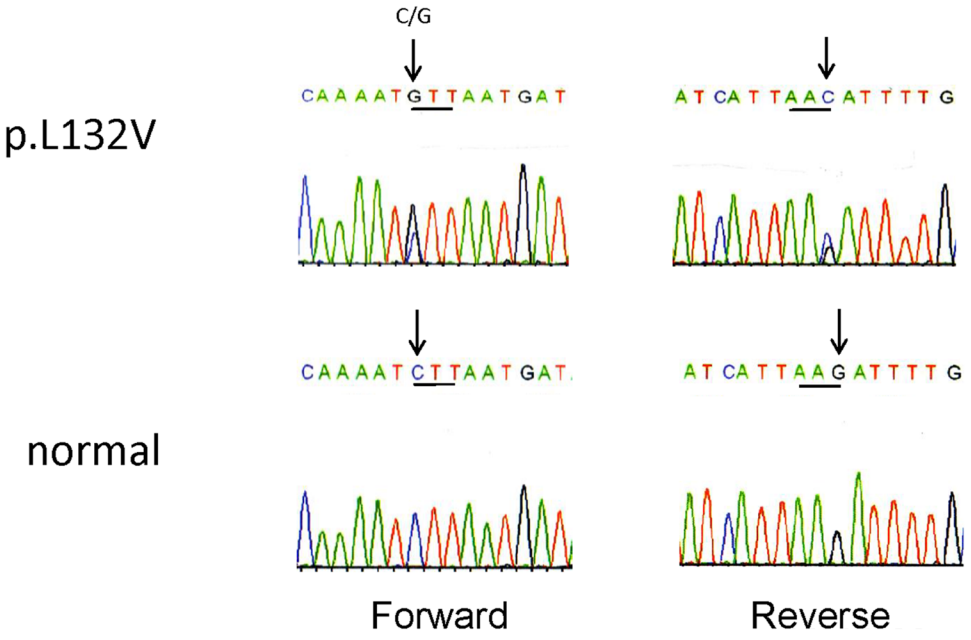

Fig. 6 Genetic mutational analysis. Bidirectional sequencing of the *KRT12* gene revealed a heterozygous missense mutation from cytosine to guanine at nucleotide position c.394 (c.394C>G), predicting substitution of a valine for a leucine at codon 132 (p.L132V, CTT>GTT) in exon 1 of all Meesmann corneal dystrophy patients

in *KRT12*, 16 were found in the 1A subdomain and 8 in the 2B subdomain. The mutation we report in this study (c.394 C>G, p.L132V) in the *KRT12* gene is located in the 1A subdomain (helix initiation motif) of the translated *KRT12* protein. This motif is important for intermediate filament assembly and is reported to mediate end-to-end interactions between keratin heterodimers [28]. The mutation we report has been previously reported only in one American patient with sporadic Meesmann corneal dystrophy [12]. However, this patient was asymptomatic whereas all of our patients had complaints of dry eye symptoms, foreign body sensation and photophobia (Table 1), suggesting that Meesmann corneal dystrophy patients with identical mutations do not necessarily present with the same symptoms. Sullivan and colleagues report a Meesmann corneal dystrophy family with an unusually severe phenotype [14]; the patients presented with symptoms and impaired visual acuity from birth. The family had a p.R430P mutation in exon 6 of *KRT12*, though other reports with mutations in the same exon did not demonstrate this symptom. Thus, not only the causative genetic mutation, but also other factors such as lifestyle habits or genetic background may have a relationship with the severity of symptoms in patients with this dystrophy.

We also performed *in vivo* histological studies by utilizing both AS-OCT and IVCM. Multiple microcysts and hyperreflective material were observed by IVCM in the corneal epithelium of all patients, as found in previous reports [15, 21, 25–27]. Ogasawara and colleagues reported depth-dependent *in vivo* histology of Meesmann corneal dystrophy using IVCM. They state that although hyperreflective material and microcysts coexisted in the superficial layer of the corneal epithelium, the ratio of hyperreflective material increased in the deeper layer of the corneal epithelium [21]. We reported for the first time in Meesmann corneal dystrophy patients the presence of subepithelial nerve abnormalities, tiny punctiform hyperreflective materials (6/6 patients) and needle-like hyperreflective materials (4/6) in the corneal stromal layer, and an atrophy of Bowman's layer (3/6). These novel findings suggest that Meesmann corneal dystrophy affects not only the corneal epithelium but also the corneal stromal layer and Bowman's layer. Since we have previously reported similar confocal findings in the corneal stroma in other types of corneal dystrophies including Bowman's layer dystrophy [29], we surmise these findings to be secondary changes due to chronic inflammation of the epithelium which may be associated with a history of recurrent corneal erosion.

AS-OCT revealed diffused hyperreflectivity in the corneal epithelium and Bowman's layer in all tested patients. No other abnormalities could be detected in the remaining corneal layers. Most notably, corneal epithelium thickness maps' obtained by Fourier-domain AS-OCT showed increased thickness of the epithelial cell layer with an average thickness of 64.8μm, which is thicker compared to previously reported normal corneal epithelium thickness (53.0±2.7μm) [24]. The thickening of corneal epithelium might be a result of reactive process because of chronic inflammation.

In this study, a clear zone of the corneal epithelium was observed in one young patient only (8 years of age: Family 3, III-1) (Fig. 2). Previously, clear zones in this dystrophy' patients have been described in 6 reports [5, 9, 17, 21, 26, 30]. In 4 of these reports, patients with clear zones were younger than 40 years old, suggesting that clear zones tend

Table 3 Reported *KRT3* and *KRT12* mutations in Meesmann corneal dystrophy

Gene	Assigned position	Exon	Nucleotide	Amino acid	References	Reference number in this paper
KRT3	head domain	1	c.250C>T	p.R84W	Chen JL et al, Mol Vis 2015 1;21:1378-86	22
	2B subdomain	7	c.1493A>T	p.E498V	Szaflik JP et al, Mol Vis 2008; 14:1713-8	15
		7	c.1508G>C	pR503P	Chen YT et al, Cornea 2005; 24:928-32	11
		7	c.1525G>A	p.E509K	Irvine AD, et al. Nat Genet 1997; 16:184-7	2
KRT12	helix initiation motif	1	c.385A>G	p.M129V	Clausen I, et al. Mol Vis 2010; 16:954-60	19
		1	c.386T>C	p.M129T	Corden LD, et al. Exp Eye Res 2000; 70:41-9	6
					Nichini O, et al. Ophthalmic Genet 2005; 26:169-73	10
		1	c.389A>C	p.Q130P	Ogasawara M, et al. Am J Ophthalmol 2014; 157:93-102	21
					Corden LD, et al. Br J Ophthalmol 2000; 84:527-30	5
		1	**c.394C>G**	**p.L132V**	Aldave AJ, et al. Contemporary ophthalmology 2005; 4-12: 1-10	12
					Nishino T, et al. (Current report)	
		1	c.395T>C	p.L132P	Liao H, et al. Plos One, 2011; 6(12), e28582	20
		1	c.395T>A	p.L132H	Wang LJ, et al. Zhonghua Yan Ke Za Zhi 2007;43:885-9	13
		1	c.399T>G	p.N133K	Irvine AD, et al. Br J Ophthalmol 2002; 86:729-32	7
		1	c.403A>G	p.R135G	Nishida K, et al. Am J Hum Genet 1997; 61:1268-75	3
		1	c.404G>T	p.R135I	Nishida K, et al. Am J Hum Genet 1997; 61:1268-75	3
		1	c.404G>C	p.R135T	Irvine AD, et al. Nat Genet 1997; 16:184-7	2
					Corden LD, et al. Exp Eye Res 2000; 70:41-9	6
					Ehlers N, et al. Acta Ophthalmol 2008; 86:40-4	18
		1	c.405A>C	p.R135S	Yoon MK, et al. Br J Ophthalmol 2004; 88:752-6	9
		1	c.409G>C	p.A137P	Takahashi K, et al. Jpn J Ophthalmol 2002; 46:673-4	8
		1	c.419T>G	p.L140R	Nishida K, et al. Am J Hum Genet 1997; 61:1268-75	3
		1	c.419T>A	p.L140Q	Ogasawara M, et al. Am J Ophthalmol 2014; 157:93-102	21
		1	c.427G>C	p.V143L	Irvine AD, et al. Nat Genet 1997; 16:184-7	2
		1	c.427G>T	p.V143L	Neilsen K, et al. Cornea 2008; 27:100-2	16
	2B subdomain	6	1222ins27bp	p.400ins9	Yoon MK, et al. Br J Ophthalmol 2004;:88:752-6	9
		6	c.1276A>G	p.I426V	Colemann CM, et al. Am J Ophthalmol 1999; 128:687-91	4

Table 3 (continued)

Gene	Assigned position	Exon	Nucleotide	Amino acid	References	Reference number in this paper
		6	c.1277T>G	p.I426S	Nichini O, et al. Ophthalmic Genet 2005; 26:169-73	10
		6	c.1285T>G	p.Y429D	Nishida K, et al. Am J Hum Genet 1997; 61:1268-75	3
		6	c.1286A>G	p.Y429C	Chen YT, et al. Cornea 2005; 24:928-32	11
		6	c.1288_1293delins6bp	p.R430_R431delins-SP	Chen JL, et al. Mol Vis 2015 1;21:1378-86	22
		6	c.1289G>C	p.R430P	Sullivan LS, et al. Mol Vis 2007; 13:975-80	14
		6	c.1298T>G	p.L433R	Seto T, et al. Jpn J Ophthalmol 2008; 52:224-6	17

to be found in the corneal epithelium of young patients. The corneal epithelia of the older members of the same family as the 8-year-old patient did not contain any clear zones, and neither did the patients from other families. However, the clinical significance and precise mechanisms of clear zone progression remain unclear [21].

In conclusion, we identified a heterozygous genetic mutation (c.394 C>G, p.L132V) in the *KRT12* gene in patients with inherited Meesmann corneal dystrophy. This mutation is novel in Japanese patients with Meesmann corneal dystrophy and is independently reported in an American Meesmann corneal dystrophy patient, confirming the pathogenicity of this mutation. The location of the *KRT12* p.L132V and high concentration of other mutations in the same domain of the *KRT12* gene confirmed the functional importance of the helix initiation motif of this gene. AS-OCT and IVCM proved to be useful tools for observing corneal epithelial layer pathology in this dystrophy. Furthermore, IVCM revealed corneal stromal layer pathological changes not previously reported in this dystrophy. Further studies using AS-OCT and IVCM in different Meesmann corneal dystrophy families with different genetic mutations may elucidate the pathogenesis of this disease.

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