A novel mutation in gelatinous drop-like corneal dystrophy and functional analysis

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Abstract
We identified a novel mutation of the tumor-associated calcium signal transducer 2 (TACSTD2) gene in a Japanese patient with gelatinous drop-like corneal dystrophy (GDLD). Genetic analysis revealed a novel homozygous mutation (c.798delG, which may result in frameshift mutation p.Lys267SerfsTer4) in the TACSTD2 gene. This mutated gene was devoid of its original function in helping the claudin (CLDN) 1 and 7 proteins transfer from the cytoplasm to the plasma membrane.

Introduction
Gelatinous drop-like corneal dystrophy (GDLD; OMIM:204870) is a rare corneal dystrophy. Most affected patients are Japanese, and the estimated incidence is 1/33,000 in Japan1. GDLD is an autosomal recessive disease characterized by the deposition of amyloid in the subepithelial region of the bilateral corneas. As amyloid deposition increases and corneal neovascularization covers the corneal surface, visual acuity becomes severely impaired. Repeated lamellar or penetrating keratoplasty is frequently required for most patients. Using positional cloning, we successfully identified the disease-causing gene, tumor-associated calcium signal transducer 2 (TACSTD2;NM_002353), thereby enabling us to investigate the molecular bases of GDLD2-4. To date, 31 different GDLD-causing alterations of the TACSTD2 gene (11 missense, 7 nonsense, and 13 frameshift mutations) have been reported to our knowledge5-16. In this study, we identified a novel homozygous frameshift mutation in the TACSTD2 gene in a Japanese family with GDLD and evaluated the pathogenic effect of the mutation.

Materials and methods
All experimental procedures for the sequencing analysis were approved by the Institutional Review Board for Human Studies at Kyoto Prefectural University of Medicine (approval number RBMR-G-148-1). All experimental procedures for the functional analysis were approved by the Institutional Review Board for gene recombination at Osaka University (approval number 2973). Prior informed consent was obtained from the investigated pedigree member after a detailed explanation of the study protocols, and this study was performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

Genomic DNA was extracted from peripheral blood. Polymerase chain reaction (PCR) was performed with a primer pair against TACSTD2 (M1S1-F-2; 5′-CCT GCA GAC CAT CCC AGA C-3′, M1S1-R-2; 5′-CAG GAA GCG TGA CTC ACT TG-3′), which fully covered the coding region of this gene. The PCR product was purified and bidirectionally sequenced using a Big-Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) in a 20 μl reaction buffer containing a 2× sequencing mixture and either of the above primers. After ethanol precipitation, the sequence products were electrophoresed on an automated capillary sequencer (Genetic Analyzer; Applied Biosystems). To further confirm the mutation detected by the above sequencing.
analysis, primer extension analysis was performed using a commercial kit (Applied Biosystems, Inc.) for the 798th nucleotide of the \textit{TACSTD2} gene coding region in the normal volunteer and patient with the forward primer.

We constructed lentivirus plasmid vectors that harbor the coding region of the \textit{CLDN1} or \textit{CLDN7} gene. Then, we coinfected HeLa cells not expressing the CLDN1, CLDN7 and TACSTD2 proteins with those lentivirus vectors and used the Tet-on system to overexpress the wild-type or mutant \textit{TACSTD2} gene in an inducible manner. First, HeLa cells were seeded at a density of 2.5 x 10^5 cells per well in a six-well plate and infected with the lentivirus vector expressing the \textit{CLDN1} or \textit{CLDN7} gene. Four days after the infection, drug selection was performed with 0.5 μg/ml puromycin for 2 weeks. Second, we coinfected those HeLa cells with the lentivirus vector expressing the wild-type or mutated \textit{TACSTD2} gene and plenti3.3/TR. Drug selection was performed with 2 μg/ml blasticidin and 500 μg/ml G418 for 2 weeks. The drug-selected HeLa cells, which should express the \textit{CLDN1} or \textit{CLDN7} genes under the control of the CMV promoter as well as the \textit{TACSTD2} gene under the control of a tetracycline-inducible promoter, were seeded on a collagen-coated culture slide (Nunc 177402 Lab-Tek Chamber Slide System with Cover Glass Slide Sterile, Thermo Fisher Scientific Inc.) at a density of 1 x 10^4 cells per well. Twenty-four hours after seeding, the cells were induced with 1 μg/ml tetracycline for 24 h. After the induction, the cells were fixed with 4% paraformaldehyde, counterstained with Hoechst 33342 dye and mounted with a commercial mounting medium (ProLong® Gold Antifade Mountant, Thermo Fisher Scientific Inc.). The cells were examined under a fluorescent confocal microscope (ELYRA S.1 / LSM710, Carl Zeiss, Oberkochen, Germany) and photographed under the Tet-on system to overexpress the wild-type or mutant \textit{TACSTD2} gene.

The patient was a 44-year-old Japanese male at the time of his first admission to our hospital. His parents were first cousins (Fig. 1a). He had already undergone several penetrating keratoplasty (PKP) surgeries prior to admission (Fig. 1b). He demonstrated severe amyloid deposition, mulberry deposition, and neovascularization, and decreased epithelial barrier function, which are the characteristic clinical manifestations of GDLD. Remarkable neovascularization and conjunctival invasion in his right eye occurred presumably as a result of cell cycle acceleration in the limbal cornea. He may present with the typical mulberry-type GDLD\textsuperscript{18}, which is not specific to this mutation\textsuperscript{19}. The \textit{TACSTD2} protein is a type I single transmembrane protein. The mutation examined was a frameshift mutation that led to premature truncation and loss of the transmembrane domain. Therefore, this frameshift mutation was thought to inactivate the \textit{TACSTD2} protein. However, the possibility of errors in translation, for

**Discussion**

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Fig. 1 Phenotype and mutation analysis. a Family tree of the proband. His parents are a first cousin marriage. He has no brothers. b In the left eye of the patient (at age 44), we found a typical mulberry-type GDLD cornea with mulberry depositions and neovascularization in the host cornea (white arrowhead) and an ulcer in the graft cornea (white arrow) before our first operation. c In the right eye of the patient (at age 44), remarkable neovascularization was observed and the graft cornea was almost covered by invading conjunctival epithelium with less than 1/4 of the corneal epithelium remaining. Presumably loss of corneal limbal stem cells caused the conjunctival invasion. d Results of direct sequencing analysis for TACSTD2 in a normal volunteer (upper) and the patient with the mutated protein (lower) sequenced in the forward (left) and reverse (right) directions are presented. Arrowheads indicate the homozygous c.798delG mutation. e One-base primer extension analysis was used to confirm the identity of the 798th nucleotide of TACSTD2 in the normal volunteer and patient. f Nucleotide and amino acid sequences of the wild-type (upper) and mutated (lower) TACSTD2 gene on both sides of the c.798delG mutation are shown. g Schematic representation of the distribution of reported TACSTD2 mutations and the domain structure of the TACSTD2 protein. An arrow indicates the 798delG mutation reported here. Missense (open circles) mutations are shown above, and nonsense (filled circles) and frameshift (filled squares) mutations are shown below. SS signal sequence, EGF epidermal growth factor-like domain, TY thyroglobulin-like domain, TM transmembrane domain, PIP2 phosphatidylinositol 4,5-bisphosphate-binding consensus sequence.
**Fig. 2** Subcellular localization of CLDNs in cells with the wild-type or p.Lys267SerfsTer4 mutated TACSTD2 gene. Without TACSTD2 gene induction, aggregated CLDN7 signals were evident, some of them seems to be in the intracellular organelles (a, c, e, g). After induction of wild-type TACSTD2 gene by tetracyclin, distribution of CLDN7 was spread with more uniformity in cytoplasm and cell membrane (b, f, q). In contrast after induction of p.Lys267SerfsTer4-mutated TACSTD2 gene, aggregated signal is apparent and the change of subcellular localization of CLDN7 was not significant (d, h, r). CLDN1 protein exhibited almost the same subcellular localization as CLDN7. Without TACSTD2 gene induction, CLDN1 signals showed aggregated pattern (i, k, m, o). After induction of wild-type TACSTD2 gene, distribution of CLDN1 showed more uniformity in cytoplasm (j, n, s). CLDN1 signals were not altered by induction of the mutated TACSTD2 gene. The aggregated bodies were apparent (l, p, t).
example, due to shifts in the reading frame, shunting of ribosomes, or skipping of stop codons, cannot be denied; therefore, we investigated the subcellular localization of CLDN1 and CLDN7 in HeLa cells. From the functional analysis, we concluded that the mutation in TACSTD2 is indeed pathological.

In conclusion, we report a novel homozygous TACSTD2 gene mutation (c.798delG that can result in a frameshift, p.Lys267SerfsTer4) in a Japanese patient with GDLD who was born to a consanguineous couple. Our functional study revealed that the mutation inactivated the TACSTD2 protein, changing the subcellular localization of the CLDN1 and CLDN7 proteins and thereby presumably disrupting the epithelial barrier function of the corneal epithelium.

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Conflict of interest
The authors declare that they have no conflict of interest.

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