washed in 0.1 M PBS, the tissues were dehydrated through an ethanol series (50, 70, 80, 90, 95, and 100%), then absolute ethanol, and finally through a 50:50 mixture of absolute ethanol and *acrylic resin* (LR White; London Resin, Reading, UK). The tissues were then embedded in LR White. Ultrathin sections (100 nmol/L) were cut on an ultratome and submitted to immunogold electron microscopy. The sections were incubated with 20% bovine serum albumin-PBS at room temperature for 20 minutes, incubated overnight with a primary antibody (goat polyclonal anti-TACSTD2) at 4°C, washed in PBS, incubated with secondary antibody (Rabbit anti-goat IgG (H+L) Gold 10 nm; British BioCell International, Cardiff, UK) at room temperature for 2 hours, and then examined ultramicroscopically by use of an electron microscope (H7000; Hitachi, Tokyo, Japan).

Immunoprecipitation Assay

The HCE-T cells were lysed in a buffer containing 10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.5 mmol/L EDTA, and a protease inhibitor mixture (Complete Mini; Roche Diagnostics). The lysate was precleared overnight with a 1/40 volume of protein A or G-sepharose (GE Healthcare UK, Buckinghamshire, UK) at 4°C, incubated at 4°C for 1 hour with a mouse monoclonal or a goat polyclonal antibody against the TACSTD2 protein, and then incubated again for 1 hour with the protein A- or G-sepharose. The captured immune complex was washed four times with the ice-cold lysis buffer. The immunoprecipitates were resuspended in Laemmli sample buffer, boiled for 5 minutes, and then subjected to Western blot analysis.

Western Blot Analysis

Proteins were separated with a commercially available 4 to 20% gradient SDS-polyacrylamide gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (FluoroTrans-W; Nihon Pall, Tokyo, Japan). The membrane was blocked in a Tris-buffered saline containing 1% skim milk and 0.05% Tween 20 and incubated overnight with primary antibodies at 4°C. After the washes, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. A chemiluminescent reagent (ECL Advance Western Blotting Detection Kit; GE Healthcare UK) was applied onto the membrane and its luminescent signal was detected by a chilled charge-coupled device camera (LAS-3000UVmini; Fujifilm, Tokyo, Japan).

Proximity Ligation Assay

In situ proximity ligation assay (PLA) analysis was performed to determine a molecular proximity between two proteins using a commercial kit (Duolink *in situ* PLA; Olink Bioscience, Uppsala, Sweden). Briefly, 10 μm cryosections were fixed with Zamboni fixative and immersed in a blocking solution. Then, the sections were incubated overnight at 4°C with a pair of primary antibodies against

two proteins raised from two different animals (eg, mouse versus goat). Then, the sections were incubated with a mixture of two secondary antibodies, respectively, labeled with two single-stranded DNA that are complementary to each other. The sections were then incubated first in a hybridization buffer, then in a ligation mixture, then in an amplification mixture, and finally in a detection mixture. After being washed with standard saline citrate buffer, the slides were air-dried, mounted, and observed by fluorescence microscopy.

Results

Tissue Localization of the TACSTD2 Protein

In the normal corneal epithelium, the TACSTD2 protein was found to be clearly localized at the cell-to-cell borders of all epithelial layers (Figure 1A). Also, in other stratified epithelia-type tissues such as conjunctiva (Figure 1B), skin (Figure 1C), pharynx (Figure 1D), esophagus (Figure 1E), uterine cervix (Figure 1J), and vagina (Figure 1K), the identical expression patterns were obtained. However, the TACSTD2 protein was not expressed in simple epithelia-type tissues such as stomach (Figure 1F), small intestine (Figure 1G), and colon (Figure 1H) or in the transitional epithelia-type tissue such as bladder (Figure 1I). Negative isotype control (normal mouse IgG$_2$a) produced virtually no fluorescent signal in any of the tissues (Figure 1L, supplemental Figure 1, A–K, see *http://ajp.amjpathol.org*).
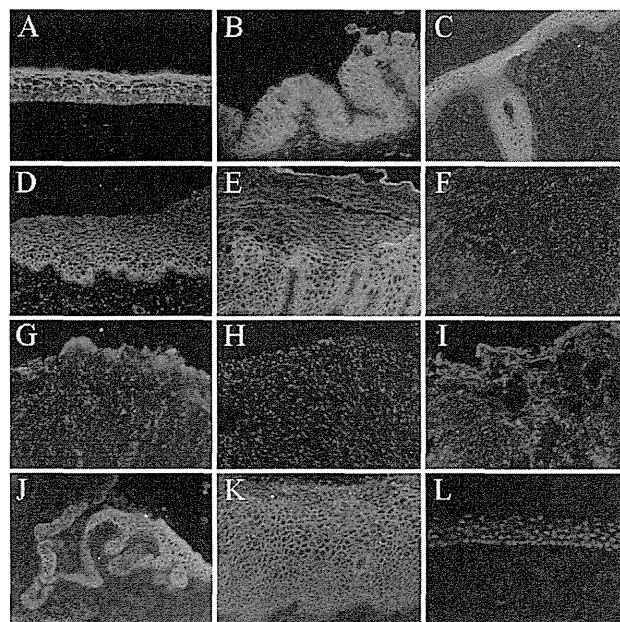

Figure 1. Expression of the TACSTD2 protein in cornea (**A**), conjunctiva (**B**), skin (**C**), pharynx (**D**), esophagus (**E**), stomach (**F**), small intestine (**G**), colon (**H**), bladder (**I**), uterine cortex (**J**), and vagina (**K**). For negative control, normal cornea was immunostained with normal mouse IgG1 or IgG2a. **L:** Green indicates the signal for the TACSTD2 protein and red indicates the signal for propidium iodide. Original magnification was ×20 (**C–K**) or ×40 (**A, B,** and **L**).
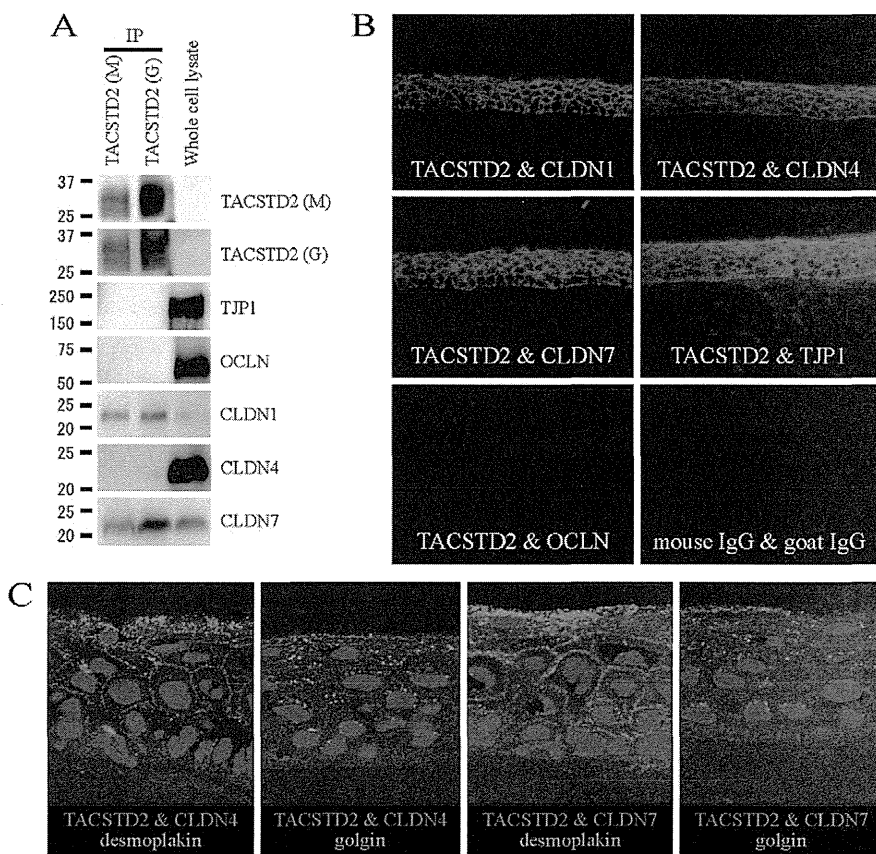
Figure 2. Relationship between the TACSTD2 protein and TJ-related proteins. **A:** HCE-T-15 cells were lysed and immunoprecipitated using an anti-TACSTD2 antibody that was raised in a mouse (M) or goat (G). The immunoprecipitants were electrophoresed and immunoblotted using antibodies against the indicated proteins. **B:** PLA analysis was performed on normal corneal tissue using pairs of antibodies against the TACSTD2 and the CLDN1, 4, 7, TJP1, and OCLN proteins. For a negative control, PLA analysis was performed using normal mouse IgG(IgG$_1$ or IgG$_2$a) and normal goat IgG. Original magnification was ×40. **C:** PLA analysis was performed using pairs of antibodies against the TACSTD2 and the CLDN4 or 7 proteins and further immunostained with antibody against desmoplakin or golgin. Red indicates PLA signal, green indicates the signal of desmoplakin or golgin, and blue indicates nucleus stained with 4′,6′-diamidino-2-phenylindole. Original magnification was ×252.

Direct Binding of the TACSTD2 Protein to CLDN1 and 7 Proteins

Previously it was reported that TACSTD2 and EpCAM proteins are serologically similar,[17,18] and our BLAST homology search revealed that the EpCAM gene is the gene most similar to the TACSTD2 gene, with 48% identity over a range of 1500 bp. The EpCAM protein has been reported to directly bind to the CLDN7 protein, and acts cooperatively with this TJ-related protein in tumor progression.[19–21] Therefore, we hypothesized that the TACSTD2 protein is spatially and functionally associated with the TJ apparatus. To verify that hypothesis, we first investigated whether the TACSTD2 protein binds to the CLDN proteins in HCE-T cells. We had identified a HCE-T subclone, HCE-T-15, which shows high epithelial barrier function[16] and express the same set of TJ-related proteins as corneal epithelium *in vivo*.[22] We used this cell line for following experiments.

Immunoprecipitation assay clearly demonstrated that the TACSTD2 protein binds to the CLDN1 and 7 proteins (Figure 2A) in the HCE-T-15 cells. The binding affinity seems relatively higher in the TACSTD2/CLDN7 complex than in the TACSTD2/CLDN1 complex. On the other hand, other TJ-related proteins such as the CLDN4, TJP1, and OCLN proteins were not coimmunoprecipitated with the TACSTD2 proteins. The immunoprecipitation experiments with varying concentrations of detergent revealed that the CLDN4, TJP1, and OCLN proteins were not coimmunoprecipitated in any of the detergent concentrations examined (data not shown).

To examination protein interaction *in vivo*, we performed the *in situ* PLA analysis using the normal corneal tissues. Positive signals were obtained between the TACSTD2 protein and either of the CLDN1, CLDN4, CLDN7, and TJP1 proteins, while no significant signal was detected between the TACSTD2 and OCLN proteins (Figure 2B). In the PLA experiment plus immunolabeling using a fluorescence-labeled antibody against desmoplakin or golgin, most of the PLA signals fall onto the plasma membrane, but only a trace amount in cytoplasm (Figure 2C). Virtually no signals were localized to the Golgi apparatus (Figure 2C). These results indicate that the association between the TACSTD2 and the CLDN proteins mainly occurred at the plasma membrane.

Decrease of Epithelial Barrier Function by the Knockdown of the TACSTD2 Gene

Next, we investigated whether the clinically observed decrease in epithelial barrier function is really a direct consequence of the ablation of the TACSTD2 gene. We constructed five lentiviral vectors expressing shRNA against the TACSTD2 gene, of which two vectors (shRNA-TACSTD2-1, 3) were found to successfully down-regulate expression of the TACSTD2 gene both in mRNA and protein level in the HCE-T-15 cells (Figure 3, A and B). The HCE-T-15 cells infected with either of these two vectors demonstrated a significantly low TER value over a 1-week period compared with their cognate controls (Figure 3C). Next, we investigated whether the knockdown of the TACSTD2 gene
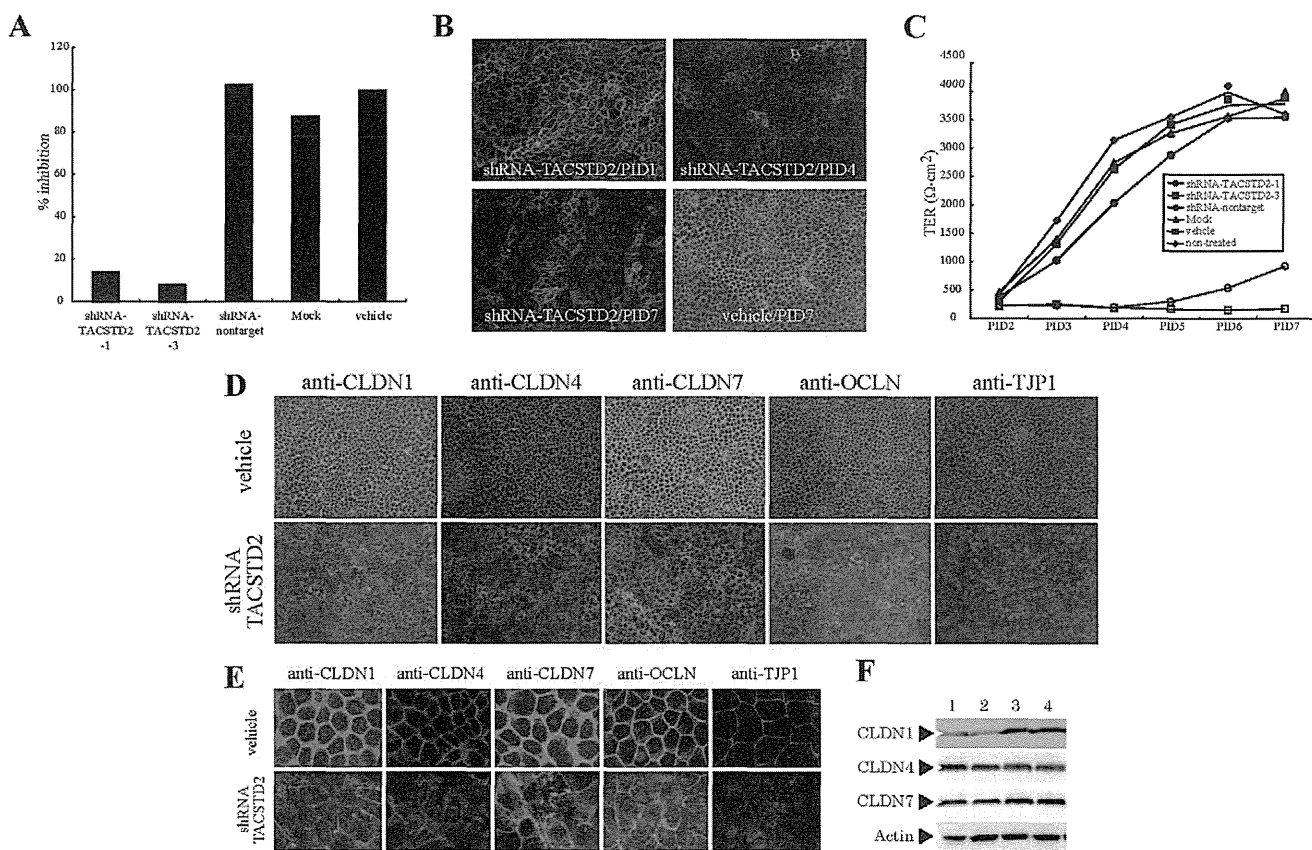
Figure 3. Knockdown of the TACSTD2 gene leads to decreased epithelial barrier function accompanied with change in expression level and subcellular localization of the TJ-related proteins in HCE-T-15 cells. **A:** Effect of the lentiviral introduction of shRNA vectors against the TACSTD2 gene on the expression of the TACSTD2 gene. The vertical bar indicates the percentile inhibition of the TACSTD2 mRNA where expression of vehicle was set to 100%. Compared with the controls (shRNA-nontarget, mock, vehicle), two shRNA vectors against the TACSTD2 gene significantly down-regulated TACSTD2 mRNA at PID 4. **B:** Effect of the lentiviral introduction of shRNA vectors against the TACSTD2 gene on the expression of the TACSTD2 protein at PID 1, 4, and 7 in the HCE-T-15 cells. Compared with the vehicle at PID 7, the expression level of the TACSTD2 protein was gradually decreased day by day in the shRNA-introduced HCE-T-15 cells. Original magnification was ×40. **C:** Effect of the lentiviral introduction of shRNA vectors against the TACSTD2 gene on the epithelial barrier function in HCE-T-15 cells. The horizontal bar indicates the number of days postinfection (PID) and the vertical bar indicates TER in $\Omega \cdot cm^2$. **D:** Effect of the lentiviral introduction of shRNA vector against the TACSTD2 gene on the expression of the CLDN1, CLDN4, CLDN7, OCLN, and TJP1 proteins. Compared with the vehicle, the shRNA-introduced HCE-T-15 cells exhibited the decreased expression in the CLDN1 and seven proteins and altered subcellular localization from plasma membrane to cytoplasm or nucleus in all of those TJ-related proteins. Original magnification was ×40. **E:** Magnified images of **D** to indicate the subcellular localization of the TJ-related proteins. **F:** Results of Western blot analysis to examine the effect of shRNA introduction on the expression of the CLDN1, CLDN4, and CLDN7 proteins. The expression levels of the CLDN1 and seven proteins were decreased in the HCE-T-15 cells introduced with shRNA against the TACSTD2 gene (lane 1, shRNA-TACSTD2-1; lane 2, shRNA-TACSTD2-3) compared with the HCE-T-15 cells introduced with shRNA-nontarget (lane 3) or mock vector (lane 4). "shRNA-nontarget" means that the lentiviral vector expressing shRNA, which was designed to have no interference with any human genes. "Mock" means the lentiviral vector without an shRNA cassette. "Vehicle" means absence of lentiviral vector infection but presence of the treatment with the same concentration of polybrene for the same period. "Nontreated" means absence of lentiviral infection as well as polybrene treatment.

in the HCE-T-15 cells has any effect on the expression of TJ-related proteins. Seven days after the infection, we found that the expression levels of the CLDN1 and 7 proteins were significantly decreased (Figure 3, D–F). In addition, the subcellular localization appeared to be changed from plasma membrane to cytoplasm in CLDN1, 4, and 7, and OCLN, and from plasma membrane to nucleus in the TJP1 protein (Figure 3, D and E).

4, and 7 genes in the HCE-T-15 cells by the introduction of shRNA expressing lentiviral vectors, all of which were found to successfully knock down mRNA of these genes (Figure 4, A–C). As expected, although the level of the decrease varied by gene, knockdown of all of those CLDN genes produced a significantly decreased TER value compared with their cognate controls (Figure 4, D–F).

Decrease of Epithelial Barrier Function by Knockdown of Either of the CLDN1, 4, and 7 Proteins

We next investigated whether the reduced expression and/or altered subcellular localization of the CLDN1, 4, and 7 proteins caused by the knockdown of the TACSTD2 gene in the HCE-T-15 cells has any effects on the epithelial barrier function. We knocked down either of the CLDN1,

TACSTD2 Protein Is Required for High Level Expression of the CLDN1 and 7 Proteins but Not for That of the CLDN4 Protein

The epithelial barrier function generally differs by the type of epithelial cells. HeLa cells demonstrated significantly low TER values even 2 weeks after they reached confluence (Figure 5A). In agreement with this observation, the endogenous expression level of the TACSTD2 protein, as
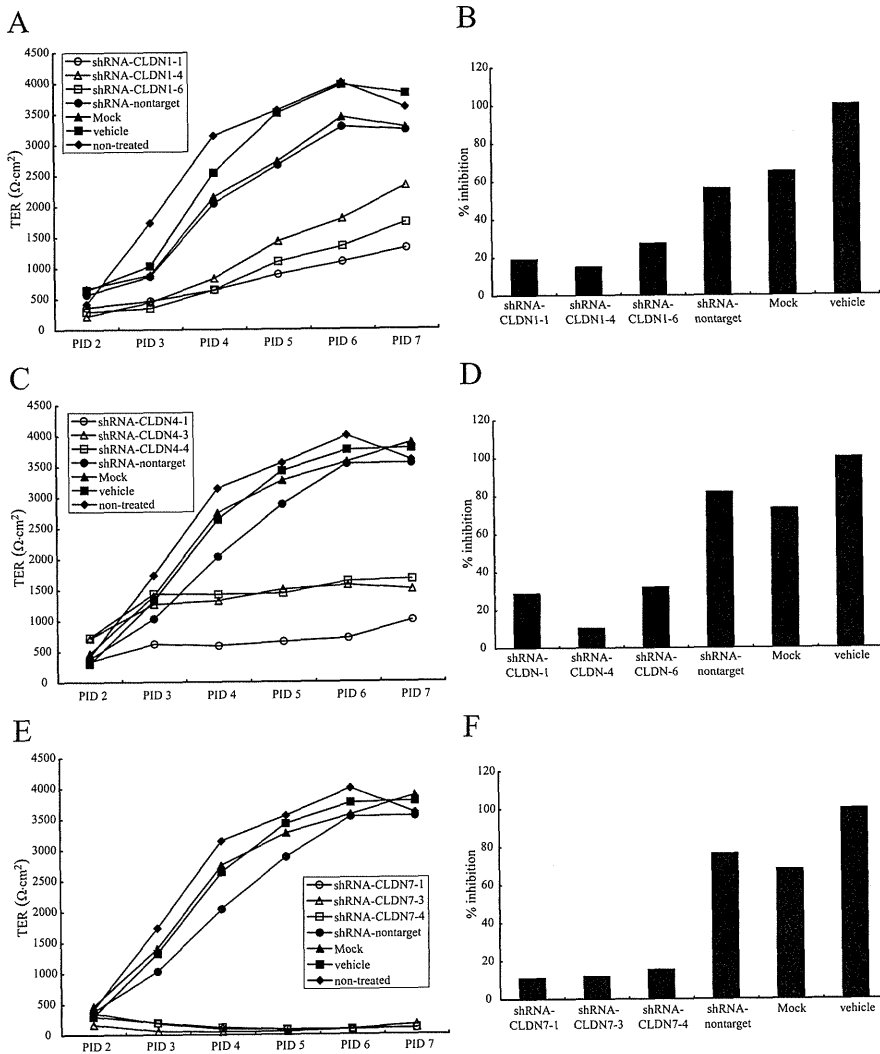
Figure 4. Knockdown of either of the CLDN1, 4, and 7 proteins significantly decreased the epithelial barrier function in the HCE-T-15 cells. HCE-T-15 cells were infected with lentiviral vectors expressing shRNA against the CLDN1 (**A** and **B**), 4 (**C** and **D**), and 7 (**E** and **F**) proteins. TER (**A**, **C**, and **E**) was measured up to seven days after the infection. The vertical bars indicate TER in $\Omega \cdot cm^2$. The horizontal bars indicate PID. Bar graphs (**B**, **D**, and **F**) demonstrate the effect of the lentiviral introduction of shRNA vectors on the expression of those genes. Vertical bar indicates the percentile inhibition of those mRNA where expression of the vehicle was set to 100%. Compared with the controls (shRNA-nontarget, mock, vehicle), for each of those genes, three shRNA vectors significantly down-regulated mRNA of their target at PID4.

well as the CLDN4 and 7 proteins, was significantly lower in HeLa cells than in HCE-T-15 cells (Figure 5B). HeLa cells were transfected with each of the lentiviral vectors expressing the CLDN1, 4, and 7 proteins. The expression level of the CLDN1 and 7 proteins was significantly lower than that of the CLDN4 protein and the subcellular localization of the CLDN1 and 7 proteins was virtually restricted to the perinuclear zone (Figure 5C). However, when HeLa cells were cotransfected with the lentiviral vectors expressing the TACSTD2 protein, the expression level of the CLDN1 and 7 proteins significantly recovered to a level as high as that of the CLDN4 protein, while the expression level of the CLDN4 protein was almost unchanged (Figure 5C). In addition, at least a recognizable amount of the CLDN1 and 7 proteins were distributed to the plasma membrane, as was found with the CLDN4 protein. These results suggest that higher expression and membrane distribution of the CLDN1 and 7 proteins require the expression of the TACSTD2 protein but the CLDN4 protein is independent of the expression of the TACSTD2 protein. This seems to be in good agreement with our immunoprecipitation results that the CLDN1 and 7 proteins bound to the TACSTD2 protein, while the CLDN4 protein did not.

TACSTD2 Protein Protects CLDN1 and 7 Proteins from the Degradation by the Ubiquitin-Proteasome System

We further investigated which mechanism causes the reduced expression of the CLDN1 and 7 proteins in the absence of the TACSTD2 protein. HeLa cells transfected with lentiviral vectors expressing CLDN1, 4, or 7 were treated with proteasome inhibitor MG-132 for 24 hours. The MG-132 treatment significantly increased the expression level of the CLDN1 and 7 proteins but had virtually no effect on the expression level of the CLDN4 protein (Figure 5, D and E). These results suggest that the TACSTD2 protein enhances the stability of the CLDN1 and 7 proteins and protects them from degradation via the ubiquitin-proteasome system.

Down-Regulation and Altered Subcellular Localization of TJ-Related Proteins in GDLD Corneas

Next, we examined the expression of the TACSTD2 and TJ-related proteins in the normal and GDLD corneas

A

B

C

anti-TACSTD2   anti-CLDN1   anti-CLDN4   anti-CLDN7
                + CLDN1 expression +CLDN4 expression +CLDN7 expression

D   +CLDN1   +CLDN4   +CLDN7   E   MG-132 (μM)
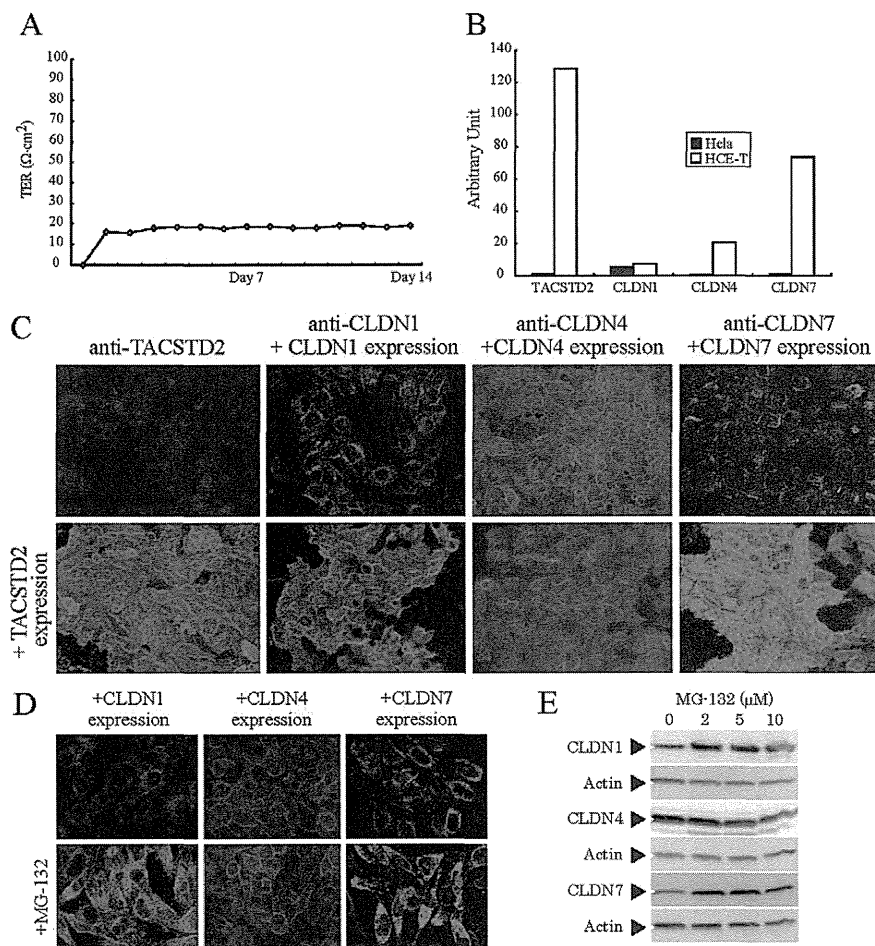   expression   expression   expression   0   2   5   10

Figure 5. Effect of the forcedly expressed TACSTD2 gene in HeLa cells on the protein expression of the CLDN1, 4, and 7 proteins. **A:** TER of HeLa cells was significantly low for more than two weeks after they reached confluence. **B:** RNA expression of TJ-related proteins in HeLa cells. Compared with HCE-T-15 cells, the expression level of the TACSTD2, CLDN4, and CLDN7 proteins was significantly low in HeLa cells. **C:** Results of immunostaining analysis against the HeLa cells transfected with the CLDN1, 4, or 7 genes together, with or without the TACSTD2 gene, using antibodies against the TACSTD2, CLDN1, CLDN4, or CLDN7 proteins. Note that the expression level of the CLDN1 and 7 proteins in the presence of the TACSTD2 gene is significantly higher than those in the absence of the TACSTD2 gene. Original magnification was ×40. **D:** Effect of the MG-132 treatment on the protein expression of the CLDN1, 4, and 7 proteins. HeLa cells transfected with the CLDN1, 4, or 7 genes were treated with proteasome inhibitor MG-132 for 24 hours. Compared with the negative control, the MG-132 treatment significantly increased the expression level of the CLDN1 and 7 proteins while that of the CLDN4 protein was virtually unchanged by the MG-132 treatment. Original magnification was ×40. **E:** Effect of the MG-132 treatment on the expression of the CLDN1, 4, and 7 proteins forcedly expressed in the HeLa cells. The MG-132 treatment significantly increased the expression level of the CLDN1 and 7 proteins in a dose-dependent manner but had virtually no effect on the expression level of the CLDN4 protein.

(Figure 6A, supplemental Figure 2A, supplemental Table 1, see *http://ajp.amjpathol.org*).[23] As easily predicted from the results of mutation analysis showing that the patients' TACSTD2 genes bear a nonsense mutation (p.118Q>X) and presumably produces a truncated TACSTD2 protein lacking a transmembrane domain (Figure 6B), the TACSTD2 protein was virtually absent in the GDLD corneas (Figure 6C, supplemental Figure 2B, see *http://ajp.amjpathol.org*). In GDLD corneas, the expression level was significantly decreased in the CLDN1, 4, and 7 proteins, and altered in their subcellular localization compared with the normal corneal epithelium (Figure 6C, supplemental Figure 2B, see *http://ajp.amjpathol.org*). TJP1 and OCLN were virtually not expressed in the GDLD corneas but were expressed at the apical side of the lateral membrane of the normal corneal epithelium (data not shown), as was found in a previous study.[10] Identical results were obtained by Western blot analysis (Figure 6D). We also examined RNA expression of those TJ-related proteins in the laser-microdissected corneal epithelial cells by qPCR analysis. Corneal epithelium of GDLD expressed mRNA of the CLDN1, 4, and 7 genes at almost the same level as normal corneal epithelium (Figure 6E). This suggests that in the corneal epithelium of GDLD, the CLDN1, 4, and 7 proteins become instable and are prone to be degraded. These results were quite consistent to the results of the *in vitro* knockdown experiments against the TACSTD2 gene

and the MG-132 treatment experiments in the CLDN 1 and 7 proteins.

Association of TACSTD2 with Desmosome

Since the tissue localization pattern of the TACSTD2 protein mimics that of desmosome, the desmosome apparatus can be another candidate for what the TACSTD2 protein functionally associates with. Double-immunostaining analysis using antibodies against the TACSTD2 protein and desmoplakin revealed an almost identical staining pattern in these two proteins, thus strongly supporting this hypothesis (supplemental Figure 3A, see *http://ajp.amjpathol.org*). However, immunoelectron microscopy analysis demonstrated that the subcellular localization of the TACSTD2 protein falls onto the plasma membrane but not the regions where the desmosome apparatus exists (supplemental Figure 3B, see *http://ajp.amjpathol.org*). In addition, *in situ* PLA analysis assessing the molecular proximity between the TACSTD2 protein and either of the three desmosome component proteins did not produce any significant signals (supplemental Figure 3C, see *http://ajp.amjpathol.org*). Therefore, we concluded that the TACSTD2 protein appears to have no spatial and functional relationship with the desmosome apparatus.
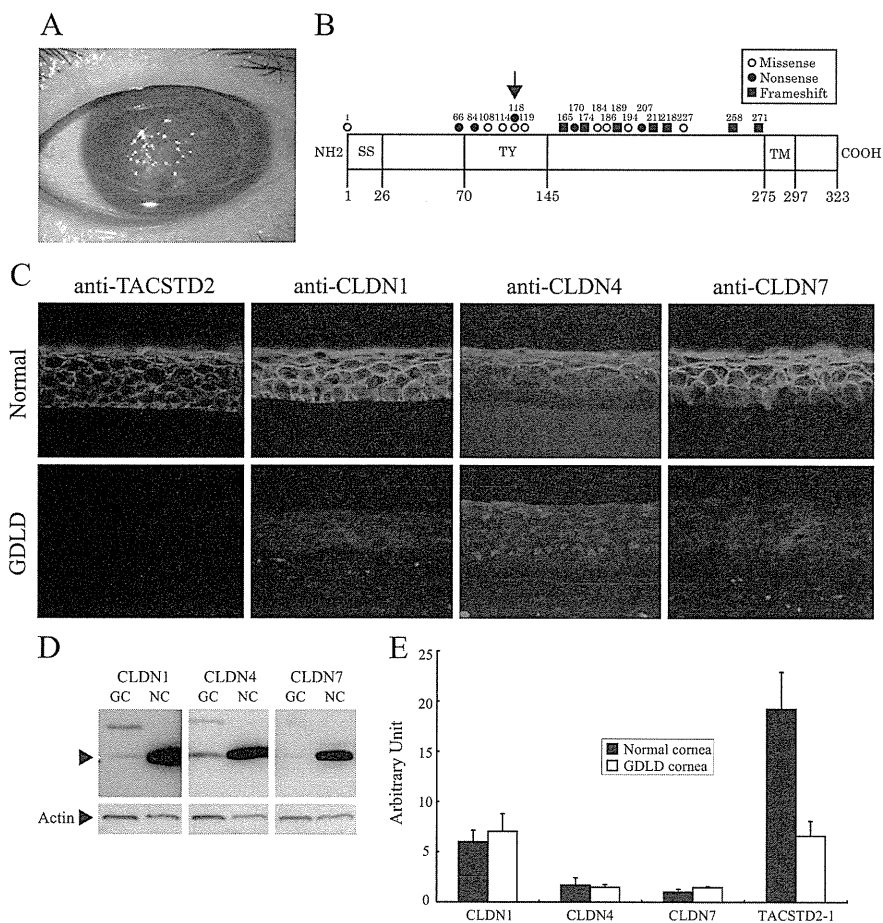
Figure 6. Expression of TJ-related proteins in the GDLD cornea. **A:** Clinical appearance of the cornea of a GDLD patient bearing a p.118Q>X nonsense mutation; grayish, protruding amyloid depositions are observed. **B:** Schematic representation of the structure of the TACSTD2 protein with mutations so far reported for the TACSTD2 gene indicated with amino acid numbers and types of mutation. The **arrow** indicates the p.118Q>X nonsense mutation. "SS" means signal sequence, "TY" means thyroglobulin repeat, "TM" means transmembrane domain, and "NH2" and "COOH" mean amino and carboxy termini, respectively. **C:** Expression of the TACSTD2, CLDN1, 4, and 7 proteins in normal and GDLD corneas. Original magnification was ×40. **D:** Results of Western blot analysis on the expression of CLDN1, 4, and 7 proteins in normal (NC) and GDLD corneas (GC). **E:** Results of qPCR analysis against the CLDN1, 4, and 7 and TACSTD2 mRNA in normal and GDLD corneas. Note that the expression level is almost identical between normal and GDLD corneas in the CLDN1, 4, and 7 mRNA.

Discussion

The TACSTD2 gene, first cloned by Fornaro et al,[24] consists of a single exon and encodes the 35,709-Da protein with a single-transmembrane domain. This gene has also been identified as a gastrointestinal tumor-associated antigen using monoclonal antibodies.[25] The TACSTD2 protein is a monomeric cell surface glycoprotein[26] expressed in many organs such as cornea, placenta, lung, kidney, pancreas, prostate, and in trophoblasts,[6,27] and at high levels in many carcinomas.[26,28,29] Although the physiological functions of this protein are still obscure, several functional domains were proposed for this molecule from its amino acid sequence. This protein contains an epidermal growth factor-like repeat, a thyroglobulin repeat,[30,31] a transmembrane region, and a phosphatidylinositol 4,5-bis phosphate-binding consensus sequence.[32] It has been suggested that the TACSTD2 protein functions as a cell-to-cell adhesion receptor in cancer cells[24] and as a calcium signal transducer.[33]

CLDNs are believed to be indispensable in forming the backbone of TJ and consist of ~24 members of a gene family with a molecular mass of ~23 kDa.[34] CLDNs have a PDZ motif at their C-terminal region, which is thought to bind to PDZ domain-possessing proteins such as TJP1, 2, and 3 and MUPP-1.[35,36] Each CLDN is expressed in a tissue- and cell-type specific fashion to achieve tissue- or cell-specific barrier function via their specific biochemi-

cal properties.[37] Certain cell types coexpress multiple CLDN genes and their combination and proportion vary by the types of cells.[38] It is theorized that multiple CLDN proteins can polymerize into paired TJ strands in a homomeric or heteromeric manner within individual strands, and in a homotypic or heterotypic manner between opposing strands.[39]

We found that the TACSTD2 protein directly binds to the CLDN1 and 7 proteins. This observation is compatible with the previous report that EpCAM, a paralogous gene of the TACSTD2 gene, directly binds to the CLDN7 protein.[20] The other TJ-related proteins, TJP1 and OCLN, were not coimmunoprecipitated with the TACSTD2 protein, even though we did obtain positive PLA signals between the TACSTD2 and all of the TJ-related proteins, except for the OCLN protein, in normal corneal tissues. Such discrepancy might be explained by the difference between these two experimental procedures. Immunoprecipitation is a method to examine whether two proteins are bound to each other, while the PLA assay is a method to examine whether the intermolecular distance between two proteins is short. Therefore, if two proteins reside very close to each other but do not directly bind, results might be different between the two methods. We theorize that the CLDN4 protein actually does not bind to, but closely resides to the TACSTD2 protein, maybe via a side-by-side or head-to-head interaction with the CLDN1 or 7

protein so that the intermolecular distance between the CLDN4 and TACSTD2 proteins is sufficiently short enough to be detected by PLA analysis. We also posit that the positive PLA signal found between the TACSTD2 and TJP1 proteins is also attributable to that same phenomenon.

As for the binding of the TACSTD2 protein to the CLDN1 and 7 proteins, we do not exactly know which amino acid sequence of the TACSTD2 protein is involved. However, a recent report[19] has shown that the EpCAM protein binds to the CLDN7 protein through interaction between the AxxxG motifs[40] that exist in their transmembrane domains. The transmembrane domain of the TACSTD2 protein is quite similar to that of the EpCAM protein with 78% identity and has the AxxxG motif at the compatible site to the EpCAM protein (Figure 7A). The CLDN1 and 7 proteins also have the AxxxG motif in their third and forth transmembrane domains, while the CLDN4 protein does not have the AxxxG motif in any of its 4 transmembrane domains (Figure 7B). Therefore, this strongly suggests that the TACSTD2 protein and either of the CLDN1 and 7 proteins bind to each other through their AxxxG motifs. In addition, absence of the AxxxG motif in the transmembrane domains of the CLDN4 protein may account for our immunoprecipitation results showing that the CLDN4 protein does not bind to the TACSTD2 protein. Sequence comparison against the entire coding sequences of 22 members of the CLDN gene family (Figure 7C) also revealed that the CLDN1 and 7 proteins have a similar amino acid sequence, while the CLDN4 protein has a quite different amino acid sequence from these two proteins, thus coinciding with our observation that the CLDN1 and 7 proteins exhibit a similar

biochemical and cytological behavior to each other, while the CLDN4 did not.

It is still unclear how the TACSTD2 protein functionally associates with the CLDN1 and 7 proteins. There seem to be at least three possibilities. First, the TACSTD2 protein acts as an anchor protein for the proper localization of the CLDN proteins at the plasma membrane. Second, the TACSTD2 protein stabilizes and prevents the CLDN proteins from degradation. Third, the TACSTD2 protein acts as a transporter in the membrane trafficking process of the CLDN proteins. We think that our time-course data in the TACSTD2 knockdown experiments may shed a light on the answers. Four days after the lentiviral infection, the TACSTD2 protein was significantly knocked down while all of the examined TJ-related proteins were virtually not affected, both in their expression level and subcellular localization. However, seven days after the infection, the expression level of the CLDN1 and 7 proteins were decreased along with altered subcellular localization in all of the TJ-related proteins examined in this study. These results may be discrepant to the first possibility, yet they do seem to be concordant with the second or third possibility. The results of the MG-132 treatment experiment strongly support the second possibility, but we feel that the third possibility is still likely since the ablation of the TACSTD2 gene in HCE-T cells altered the subcellular localization of the CLDN1 and 7 proteins. Further experiments will be needed to fully uncover the exact mechanism.

In summary, our current study has unveiled the yet-unlinked pieces of the puzzle between the loss of function mutation of the TACSTD2 gene and the decreased epithelial barrier function in GDLD corneas. The pathological processes that occur in GDLD corneas appear to be that mutation of the TACSTD2 gene firstly causes the loss of function of this protein, secondly leads to decreased expression and altered subcellular localization of the CLDN proteins, thirdly causes decreased expression and altered subcellular localization of the TJP1 and the OCLN proteins, and finally causes significant impairment in the formation and maturation of the TJ. Although we currently do not have any information for other types of epithelia than corneal epithelium, we theorize that the TACSTD2 gene has a general role in the formation of the TJ over a broad spectrum of the stratified epithelia. However, it is still unclear why organs other than the cornea that are covered by stratified epithelia do not demonstrate the disease phenotype in GDLD patients. This question should be considered as the next major challenge to be thoroughly resolved for the complete understanding of the pathogenesis of GDLD. We hope that our current study will contribute to future investigations aimed at an improved understanding the pathogenesis of GDLD, as well as the development of better treatments for this disease.
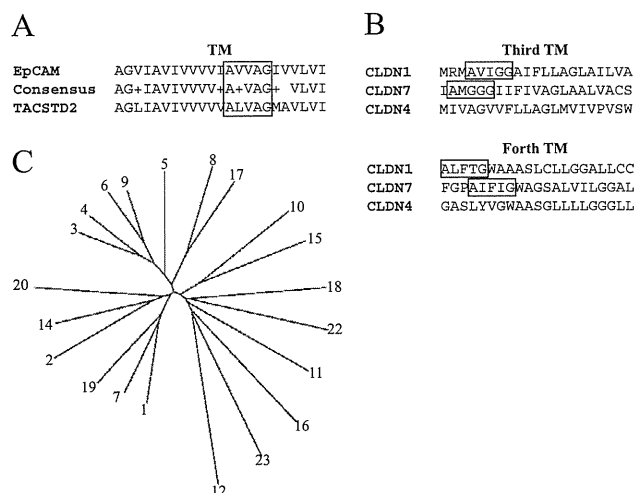

Figure 7. Sequence comparison between the TACSTD2 and the EpCAM proteins and among the CLDN1, 4, and 7 proteins in their transmembrane domains. **A:** Both the TACSTD2 and the EpCAM proteins have an AxxxG motif (enclosed by a square) in their transmembrane (TM) domain. **B:** CLDN1 and 7 proteins have 2 AxxxG motifs in their third and forth transmembrane domains, while the CLDN4 protein does not have an AxxxG motif in any of its 4 transmembrane domains. **C:** Schematic representation of a molecular phylogenetic *tree* among 22 members of the CLDN gene family depicted in an unrooted N-J tree image. Note that the CLDN1 and 7 genes are quite similar over their entire coding regions while the CLDN4 gene is quite different from these two genes.

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Gelatinous Drop–Like Corneal Dystrophy

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Abstract: Gelatinous drop–like corneal dystrophy (GDLD) is a rare autosomal recessive disorder, clinically characterized by grayish corneal deposits of amyloid and by severely impaired visual acuity. Most patients require corneal transplantation. We identified the gene responsible for GDLD, *tumor-associated calcium signal transducer 2 (TACSTD2)*, by positional cloning and detected 4 disease-causing mutations in Japanese patients with GDLD. During the positional cloning process, strong linkage disequilibrium was observed between GDLD and some markers in the critical region. More than 90% of GDLD patients possessed the same haplotype with a Q118X mutation in *TACSTD2*. This may be the result of a founder effect and reflects that most GDLD patients are Japanese. *TACSTD2* deleterious mutations resulted in destabilized tight junction proteins, including claudins, ZO-1, and occludin. These findings may explain why the corneal epithelium barrier function is impaired in GDLD patients.

Key Words: gelatinous drop–like corneal dystrophy, positional cloning, Q118X mutation, *TACSTD2*, tight junction

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G elatinous drop–like corneal dystrophy (GDLD; OMIM: 204870) is a relatively rare corneal dystrophy disease with an autosomal recessive trait. GDLD was first reported in 1914 by Nakaizumi in Japan.[1] The incidence is reportedly 1 in 300,000 in Japan; however, in other countries, GDLD is a very rare disease.[2] It is characterized by the deposition of amyloid material in the subepithelial space of the cornea, which causes blurred vision and photophobia from the first decade of life. Eventually, raised gray gelatinous masses severely impair visual acuity, and lamellar or penetrating keratoplasty is required for most patients. Unfortunately, the same condition with amyloid deposition often develops in the transplanted cornea within several years, and additional keratoplasty is needed. Immunohistochemical studies have revealed that amyloid depositions contain the lactoferrin protein, which had been considered a disease-causing gene. However, this candidate approach was not successful. Thus, we undertook a positional cloning approach to identify the gene responsible for GDLD.

LINKAGE ANALYSIS

To identify the gene responsible for GDLD, we used a strategy of conventional positional cloning, called "homozygosity mapping."[3] For this method, the samples must be obtained from consanguineous families; thus, the total number of samples may be small. In our study, we could map the locus from only 10 Japanese GDLD families.[4] After examining 63 markers distributed throughout the genome, we found that all 13 affected members and none of the 11 unaffected members from the 10 consanguineous GDLD families studied were homozygous at the D1S220 locus on the short arm of chromosome 1. This homozygosity occurs in consanguineous families because the same chromosomal region was delivered from both the paternal and maternal ancestors. Using additional markers flanking the D1S220 locus to confirm linkage, we found that all of the affected individuals were homozygous for 2 additional markers (D1S2831 and D1S2741) on the proximal side of D1S220 and for 2 markers on the distal side (D1S2869 and D1S2650). From these 5 markers, linkage analysis revealed no recombination, with LOD scores of 4.40 to 9.80; the maximum score of 9.80 was obtained at the D1S2741 locus. Haplotype analysis indicated that 3 patients were heterozygous at the D1S2890 locus proximal to D1S220, and 2 patients were heterozygous at the D1S2801 locus distal to D1S220. These cases defined a critical region for GDLD, within a 2.6-cM interval between D1S2890 and D1S2801. However, this 2.6-cM interval was still too large to perform positional cloning. We observed that 8 of 10 disease chromosomes (80%) carried the 247-bp allele at the D1S220 marker locus, whereas only 5% of the unaffected control chromosomes carried this allele. A significant linkage disequilibrium between GDLD and the D1S220 locus ($\chi^2 = 36.24$; $P < 0.001$) was observed. We also found that D1S2648 ($\chi^2 = 12.90$; $P = 0.032$) and D1S2752 ($\chi^2 = 19.77$; $P = 0.012$) showed significant linkage disequilibrium, indicating that the most critical region lay between them.

POSITIONAL CLONING

To identify the gene responsible for GDLD, we subsequently isolated cosmid and bacterial artificial clones approximately covering the 400-kb critical region between D1S2752 and D1S220 and then performed DNA sequencing experiments using a shotgun cloning method (Fig. 1). Computer analysis of genomic DNA sequences indicated that 6 expressed sequence tags and a single known gene, *tumor-associated calcium signal transducer 2 (TACSTD2;* also known as *M1S1)*, consisting of a single exon, were located in the region. Northern blot analysis revealed that *TACSTD2* was expressed in the cornea, as well as in the kidney, lung,
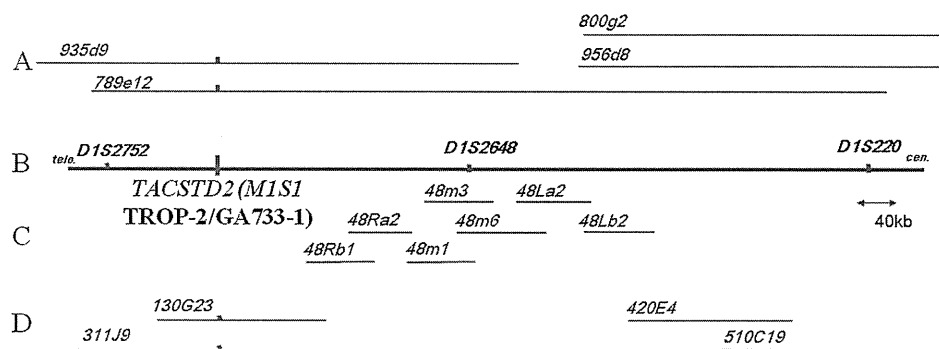
FIGURE 1. Physical map of the GDLD region on chromosome 1. A, YAC clones. B, Positions of DNA markers and *TACSTD2*. C, Overlapping cosmid clones. D, Overlapping bacterial artificial clones. Reprinted with permission from Tsujikawa et al.[5]

placenta, pancreas, and prostate gland, suggesting that this gene may be a candidate for harboring mutations responsible for GDLD. We amplified a 1.8-kb fragment, covering the entire coding region of *TACSTD2*, from genomic DNA (*TACSTD2* is a single exon gene) isolated from members of 20 Japanese GDLD families and determined DNA sequences. All 26 affected members of these families were homozygotes or compound heterozygotes for the mutations shown in Table 1. The most commonly detected mutation was a C to T transition at nucleotide 352, replacing a glutamine at codon 118 with a stop codon (Q118X).[5] Affected members from 16 of 20 GDLD families were homozygotes for this mutation. In addition, a patient from another family carried this mutation on one allele and a different mutation on the other. Thus, the Q118X mutation accounted for 82.5% (33 of 40) of the disease alleles present in our panel of GDLD families. All 33 alleles carried the major disease haplotype (Table 1), indicating that the Q118X mutation is the major Japanese GDLD mutation and reflects the linkage disequilibrium reported previously. This nucleotide alteration was not observed in 100 normal healthy Japanese people, and we found 2 other nonsense mutations and a frameshift mutation. Thus, we concluded that the *TACSTD2* gene is responsible for GDLD.

LINKAGE DISEQUILIBRIUM

After the identification of the responsible gene, we and other groups reported on the genotype spectrum in Japanese and other GDLD patients around the world.[6–8] We also performed mutation analysis of new additional patients using a protein truncation test.[9] Protein truncation tests use in vitro translation and sodium dodecyl sulfate polyacrylamide gel electrophoresis to detect the truncated protein product from the DNA of patients. Fifteen new families were analyzed and found to have the homozygous Q118X mutation. Haplotype analysis using nearby polymorphic markers in other patients indicated that this Q118X mutation is a Japanese founder mutation and reflects linkage disequilibrium. This may explain why most GDLD patients are Japanese and few cases have been reported in other countries. In Japanese patients, 90% of the disease chromosomes have this major mutation. This allelic homogeneity is an interesting phenomenon in Japanese corneal dystrophies. In addition, we also reported allelic homogeneity as a result of the founder effect in other corneal dystrophies in Japan.[10,11]

ATYPICAL CASES

In GDLD, clinical variability and atypical cases have been reported.[12] An important question is whether these atypical cases are caused by genetic background differences, including allelic or locus heterogeneities. To address this, we performed genetic analyses of 4 Japanese families who had bilateral corneal amyloidoses.[13] All families included a patient whose clinical features alone could not be used to diagnose GDLD. In 1 family, obvious clinical differences were observed between 2 members who had corneal amyloidosis. Members from 3 families had atypical amyloidosis that had not been initially diagnosed as GDLD (Fig. 2). Sequence analysis revealed that all the patients possessed a homozygous Q118X mutation in *TACSTD2*. There were no differences in the entire sequence of *TACSTD2* in these patients compared with other GDLD patients. Moreover, the genotyping of polymorphic markers near the *TACSTD2* gene revealed that these patients shared the same founder chromosome along with *TACSTD2* (Fig. 3). Therefore, even in atypical cases, GDLD patients carry the same genetic background around *TACSTD2*.

FUNCTIONAL ANALYSIS

The identification of the responsible gene for GDLD enabled us to investigate the pathogenic mechanisms of GDLD using reverse genetic methods. The function of the encoded protein, TACSTD2, is not well understood, but several potential modification sites within the molecule have been suggested (Fig. 4). TACSTD2 contains an epidermal growth factor–like repeat and a thyroglobulin repeat. This structure suggests that TACSTD2 is a cell adhesion molecule.

TABLE 1. Haplotype and Disease-Causing Mutations in GDLD Patients Originally Identified in 1999

Mutation	Frequency (%)	Haplotype		
		D1S2752	D1S2684	D1S220
Q118X	82.5	204	285	247
632delA	7.5	210	285	235
Q207X	5.0	236	273	235
S170X	5.0	204	285	247

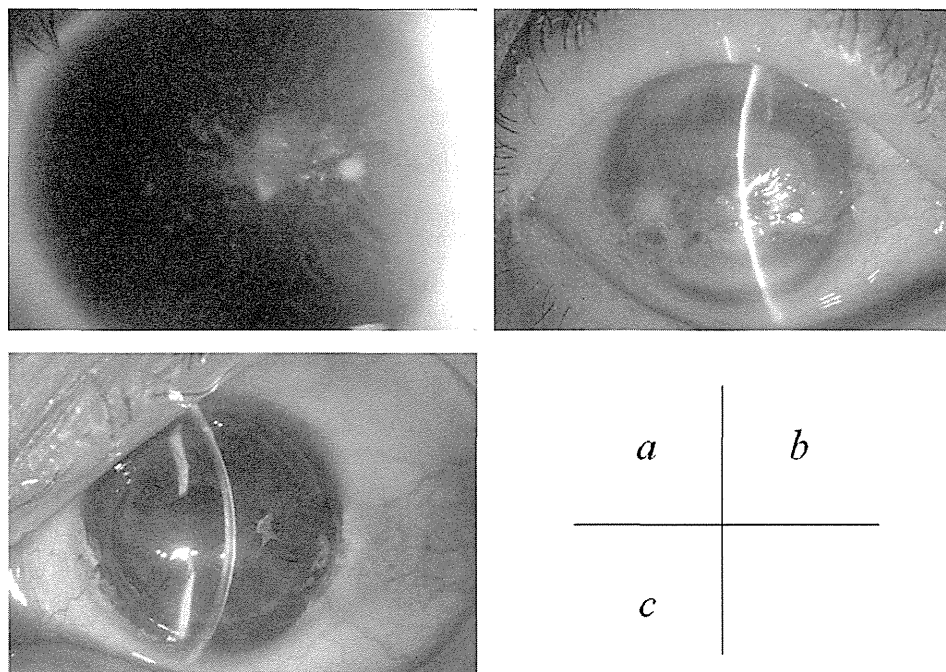Reprinted with permission from Tsujikawa et al.[5]

FIGURE 2. Phenotypic variability (clinical heterogeneity) among families with GDLD. All patients possess the Q118X mutation.
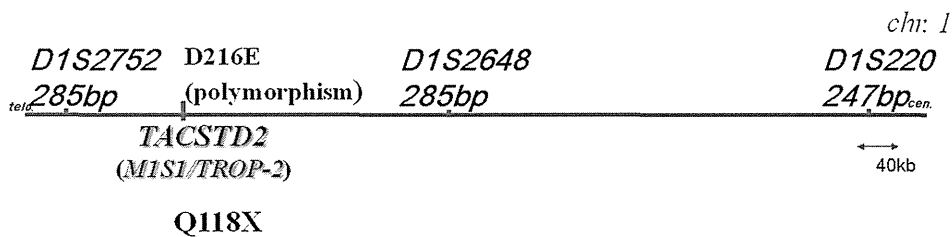

FIGURE 3. Haplotype of the founder chromosome of Japanese GDLD. D216E polymorphism is an intragenetic marker.

Furthermore, we have observed that TACSTD2 can induce hemophilic binding (unpublished data). This is interesting because the corneal epithelium of GDLD patients has a significantly increased permeability for fluorescence.[14] Additionally, the apical side of the corneal epithelium of

FIGURE 4. Schematic representation of the structure of TACSTD2. EGF, epidermal growth factor–like repeat; PIP2, phosphatidylinositol 4,5-bisphosphate–binding sequence; SS, signal sequence; TH, thyroglobulin repeat; TM, transmembrane domain. Arrows indicate the locations of the reported mutations.

GDLD exhibited loosened cell–cell junctions and an increased number of scarred cells compared with normal cornea.[14] Recently, it was shown that TACSTD2 can bind to the proteins claudin-1 and claudin-7 and stabilize these in corneal cells.[15] In the absence of *TACSTD2* expression, there is a change in the subcellular localization of tight junction–related proteins, including claudin-1, claudin-4, claudin-7, ZO-1, and occludin, leading to impaired corneal epithelial barrier function. However, TACSTD2 has a cytoplasmic tail with a phosphatidylinositol 4,5-bisphosphate–binding consensus sequence. Thus, TACSTD2 is thought to be a calcium transducer, although how this function may play a role in GDLD remains unknown.
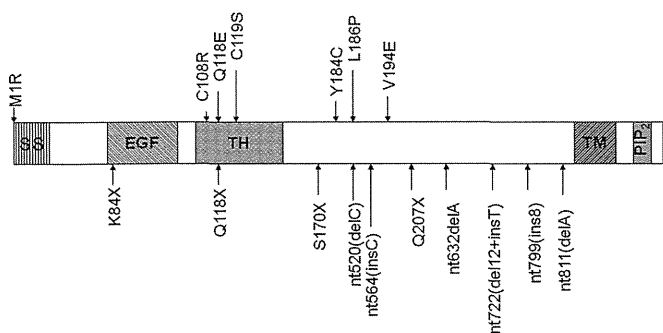
CONCLUSIONS

Using positional cloning, we have identified *TACSTD2*, the gene responsible for GDLD. The major mutation identified was Q118X in *TACSTD2* for the majority of Japanese GDLD cases. Among the Japanese GDLD families, a founder effect was observed, and this likely explains why GDLD is so dominant among the Japanese population. Even in atypical cases, the founder chromosomal region was preserved.

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