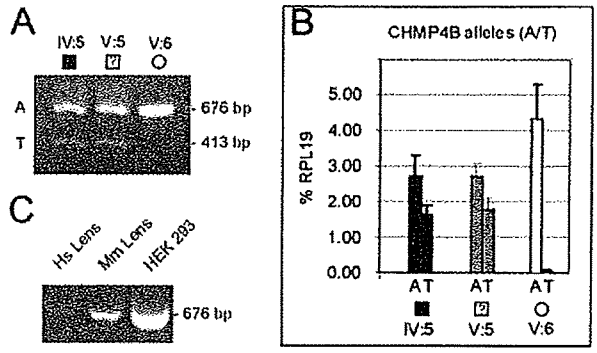

Figure 3. RT-PCR analysis of *CHMP4B* transcripts in peripheral blood leukocytes (PBLs) and eye lens. *A,* Agarose-gel electrophoresis showing nested amplification products of *CHMP4B* transcripts in PBL RNA from family Sk, confirming that individuals IV:5 and V:5 are heterozygous for the mutant T allele (413 bp), whereas individual V:6 is homozygous for the wild-type A allele (676 bp). PBL RNA was purified using the Versagene kit (Gentra), reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad), and PCR amplified as above with three primers (StartF, nested T-alleleR, and EndR) (table 3). *B,* Quantitative amplification of *CHMP4B* transcripts from PBL RNA with allele-specific primers (StartF + A-alleleR, or StartF + T-alleleR) (table 3) showing the relative levels of wild-type (A allele) and mutant (T allele) transcripts in individuals IV:5, V:5, and V:6 from family Sk. RT-PCR products were amplified in a 10-fold dilution series (in triplicate) by use of the iQ SYBR Green Supermix in an iCycler fitted with a MyiQ single-color real-time PCR detection system (Bio-Rad). Allele-specific *CHMP4B* transcripts were detected by melt-curve analysis and standardization against control RPL19 transcript, which was amplified separately in a similar 10-fold dilution series of the same PBL RT-PCR products by use of RPL19 forward (5′-catccgcaagcctgtgac-3′) and reverse (5′-gtgaccttctctggcattcg-3′) primers. *C,* Agarose-gel electrophoresis showing amplicons containing the entire coding region (codons 1–224) of *CHMP4B* transcripts (676 bp) from human (Hs) lens (~30 years old), mouse (Mm) lens (postnatal day 6), and HEK 293 cells. Post mortem human lenses were obtained from the Lions Eye Bank of Oregon, and RNA was extracted using TRIzol reagent (Invitrogen). Following euthanasia (CO_2 gas), mouse lenses were dissected into RNA*later* tissue preservative, and RNA was extracted using the RNAqueous kit (Ambion). RNA was extracted from cultured HEK 293 cells as for mouse lenses. RT-PCR of lens and HEK 293 RNA was performed as for PBL RNA above, with use of StartF and EndR primers (table 3), and the resulting amplicons were verified by sequencing.

within cells and release of Gag into the media as VLPs was detected by immunoblotting (fig. 6*E*). As expected on the basis of previous results,[49] expression of the truncated wild-type fragment (FLAG-CHMP4B$_{1\text{-}150}$) inhibited VLP release. Interestingly, the truncated mutant fragment (FLAG-D129V-CHMP4B$_{1\text{-}150}$) was a more potent inhibitor than truncated wild type allowing release of only 53% ± 7% (average ± SD) as much Gag in VLPs. Correspondingly, the level of Gag expression in cells expressing the mutant fragment was 1.4 ± 0.3 times that of cells expressing the wild-type fragment. In contrast, neither the wild type nor the mutant forms of full-length CHMP4B significantly inhibited Gag production or VLP release (data not shown).

Precisely how the p.D129V substitution affects the function of CHMP4B is unclear. In this study, we found that the p.D129V substitution changed the subcellular distribution and effects of CHMP4B on VLP release when the protein's acidic C-terminus was removed. Previous studies suggest that the acidic C-termini of CHMPs are regulatory domains that interact specifically with their cognate N-terminal basic domains in an auto-inhibitory manner.[46,49,50] Thus, it is possible that when CHMP4B is relieved from auto-inhibition (mimicked here by truncation), the p.D129V substitution is exposed resulting in deleterious gain-of-function effects. On the basis of expression anal-

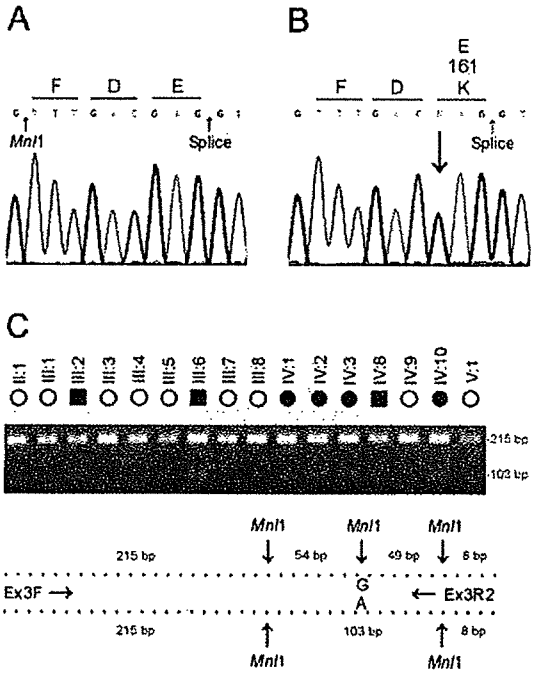

Figure 4. Mutation analysis of *CHMP4B* in the CPP3 family. *A,* Sequence trace of the wild-type allele showing translation of glutamic-acid (E) at codon 161 (GAG). *B,* Sequence trace of the mutant allele showing the heterozygous c.481G→A transition (denoted R by the IUPAC code) that is predicted to result in the missense substitution of lysine (AAG) for glutamate at codon 161 (p.E161K). *C,* Restriction-fragment–length analysis showing loss of an *MnlI* site (3′-GGAGN$_6$) that cosegregates only with affected individuals from the Japanese family[31] heterozygous for the c.481G→A transversion (103 bp). Exon 3 was amplified with PCR primers (table 3) shown in the schematic diagram and resulting amplicons (326 bp) digested (at 37°C for 1 h) with *MnlI* (5 U; New England BioLabs). Restriction fragments (>75 bp) were visualized on 2% agarose-EtBr gels.

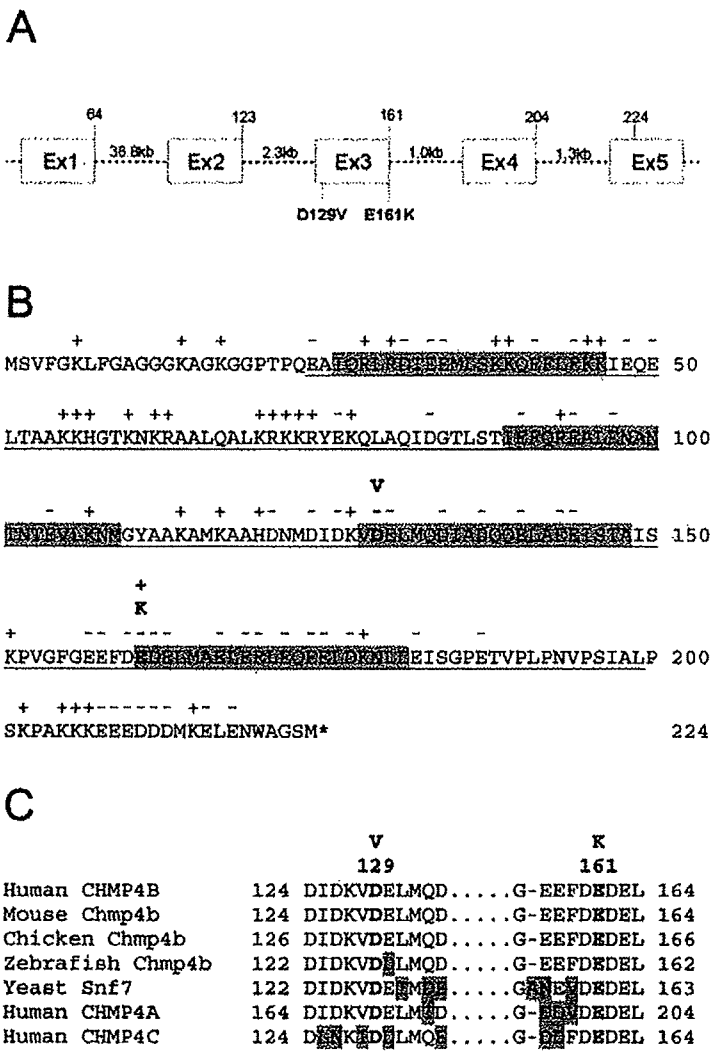

Figure 5. Gene structure and protein domains of CHMP4B. *A*, Exon organization and mutation profile of *CHMP4B*. Intron sizes are indicated (kb), and codons are numbered above each exon. *B*, Amino acid sequence of CHMP4B, showing the conserved SNF7 domain (*underlined*) of this protein family (Conserved Domain Database, pfam03357) containing at least four predicted helical domains (*grey*). The proposed p.D129V and p.E161K substitutions are predicted to be located in the C-terminal acidic half of the protein, near the start of adjacent helices within the SNF7 domain. Charged amino acids (+, −) and the translation stop codon (*) are also indicated. *C*, Amino acid sequence alignment of human CHMP4B and orthologs from other species, showing phylogenetic conservation of D129 and E161.

ysis of the N-terminal region of CHMP4A,[46] we speculate that, once unmasked, the p.D129V substitution alters the polymerization and/or membrane-binding properties of CHMP4B; however, other mechanisms cannot be excluded. Further work will be required to understand how the p.D129V change affects the behavior of intact CHMP4B. Functional expression studies are also underway to determine how the p.E161K substitution affects CHMP4B. Although little is known about the role of CHMP proteins in lens development, endosome-like compartments have been observed in the newborn mouse lens.[51] Further characterization of endosomal pathways in the lens should provide insight into the pathogenetic mechanisms linking CHMP4B dysfunction with cataractogenesis.

In conclusion, our data identify the first mutations (p.D129V, p.E161K) in a novel gene (*CHMP4B*) for inherited cataracts linked to 20q, and they suggest that gain-of-function defects in an endosome-sorting complex (ESCRT-III) subunit triggers loss of lens transparency.

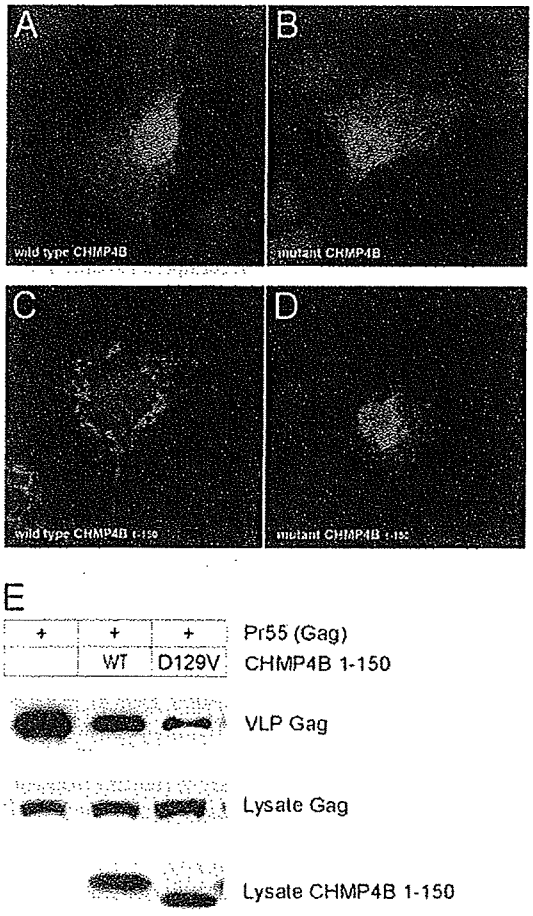

Figure 6. Transient expression of CHMP4B in cultured cells. *A–D*, Subcellular localization of CHMP4B proteins in COS-7 cells, visualized by immunostaining with FLAG antibody and epifluorescence microscopy. *A*, Full-length wild-type FLAG-CHMP4B. *B*, Full-length mutant FLAG-D129V-CHMP4B. *C*, Truncated wild-type FLAG-CHMP4B$_{1-150}$. *D*, Truncated mutant FLAG-D129V-CHMP4B$_{1-150}$. For full-length constructs, the coding sequence (codons 1–224) of human CHMP4B was PCR amplified from HeLa cDNA (Clontech) with forward (5′-gtagatctatgtcggtgt-tcgggaagctgttcgg-3′) and reverse (5′-cactcgagttacatggatccagcccagttctcc-3′) primers and then subcloned into the *Bam*HI and *Xho*I restriction sites in the poly-linker of pcDNA3.1-FLAG.[46] The D129V substitution was generated using the QuickChange mutagenesis kit (Stratagene). For truncated CHMP4B constructs, amplicons corresponding to codons 1–150 were amplified using the full-length constructs as templates and were subcloned into pcDNA3.1-FLAG as above. Plasmid DNA was prepared using the QIAprep spin kit (Qiagen), and inserts were verified by sequencing using the T7 primer. For transient expression, cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 5% fetal bovine serum (Gibco-BRL), 5% supplemented calf serum (Hyclone Laboratories), and 2 mM glutamine. Cells were transfected with expression plasmids by use of Lipofectamine 2000 reagent (Invitrogen). At 18–24 h after transfection, COS-7 cells grown on glass cover slips were fixed in 3.5% paraformaldehyde, permeabilized in 0.2% Triton X-100, and immunostained with rabbit FLAG antibody (Sigma) followed by Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). Cell nuclei were counterstained (*blue*) with DAPI (4′,6-Diamidino-2-phenylindole [Molecular Probes]). *E*, Immunoblot analysis of VLPs produced by HEK 293T cells cotransfected with plasmids encoding Gag (p24 antibody) and CHMP4B$_{1-150}$ (FLAG antibody). Top blot shows Gag recovered in VLPs, and middle blot shows Gag in cell lysates. Bottom blot shows that the levels of CHMP4B$_{1-150}$ were similar in cell lysates; however, the D129V substitution increased the electrophoretic mobility of the mutant fragment on SDS (sodium dodecyl sulfate) polyacrylamide gels compared with its wild-type counterpart. For VLPs, HEK 293T cells were transfected with 4 μg pCMV55 encoding HIV Gag, alone or together with 1μg of the indicated CHMP4B construct. At 18–24 h after transfection, media containing VLPs was harvested and clarified by passing through a 0.45 μm filter. VLPs were pelleted by centrifugation (3 h) through a 20% sucrose cushion at 26,000 rpm in SW41 Ti rotor (Beckman Coulter). VLPs and cell lysates were resuspended in sodium dodecyl sulfate (SDS) sample buffer, were separated by SDS-polyacrylamide gel electrophoresis, and then were analyzed by immunoblotting using rabbit antibody against p24, the capsid domain of HIV Gag, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and the SuperSignal West Pico chemiluminescence detection kit (Pierce). Immunoblot signals were quantified using the Odyssey Infrared Imaging System (Li-Cor Bioscience).

Acknowledgments

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

ClustalW multiple sequence alignment, http://www.ebi.ac.uk/clustalw/http
Conserved Domain Database (CDD), http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml
Entrez Protein database, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein
Généthon, Marshfield, and deCODE genetic linkage maps, http://www.ncbi.nlm.nih.gov/genome/guide/human/
LINKAGE/MLINK, http://linkage.rockefeller.edu/soft/
NCBI, http://www.ncbi.nlm.nih.gov/index.html
NCBI Map Viewer, http://www.ncbi.nlm.nih.gov/mapview/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.govOMIM
SNP database (dbSNP), http://www.ncbi.nlm.nih.gov/projects/SNP/
UniGene, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene

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Mutations in CD96, a Member of the Immunoglobulin Superfamily, Cause a Form of the C (Opitz Trigonocephaly) Syndrome

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The C syndrome is characterized by trigonocephaly and associated anomalies, such as unusual facies, psychomotor retardation, redundant skin, joint and limb abnormalities, and visceral anomalies. In an individual with the C syndrome who harbors a balanced chromosomal translocation, t(3;18)(q13.13;q12.1), we discovered that the *TACTILE* gene for CD96, a member of the immunoglobulin superfamily, was disrupted at the 3q13.3 breakpoint. In mutation analysis of nine karyotypically normal patients given diagnoses of the C or C-like syndrome, we identified a missense mutation (839C→T, T280M) in exon 6 of the *CD96* gene in one patient with the C-like syndrome. The missense mutation was not found among 420 unaffected Japanese individuals. Cells with mutated CD96 protein (T280M) lost adhesion and growth activities in vitro. These findings indicate that CD96 mutations may cause a form of the C syndrome by interfering with cell adhesion and growth.

The C (Opitz trigonocephaly) syndrome (MIM %211750) is a malformation syndrome of unknown cause, and its mode of inheritance has been suggested to be autosomal recessive. The syndrome comprises trigonocephaly and associated anomalies, such as unusual facies, wide alveolar ridges, multiple buccal frenula, limb defects, visceral anomalies, redundant skin, psychomotor retardation, and hypotonia.[1,2]

Recently, Bohring et al.[3,4] suggested the delineation or existence of a severe form of the C syndrome (the C-like syndrome, or Bohring-Opitz syndrome [MIM 605039]). More recently, Osaki et al.[5] reported on a newborn infant who had many clinical features similar to those of the C-like syndrome but did not have exophthalmoses, which has been regarded as a hallmark of the C-like syndrome. They suggested that the manifestations in this patient are a further indication of overlap between the C-like syndrome and the C syndrome. Thus, it is controversial whether there is (1) a gradient of spectrum in the C syndrome, from the mild form (C syndrome) to the severe form (C-like syndrome), or (2) genetic heterogeneity among the patients with the C syndrome.

In addition, various chromosomal abnormalities, especially those that include chromosome 3, have been reported in patients originally described as having the C syndrome.[2] These include 3p monosomy,[6] distal 3p trisomy,[7] 3q trisomy,[8] distal 3q trisomy with deletion of distal 3p,[9] and inversion in chromosome 3.[10] Although these cases might be removed from the C syndrome because they involve chromosome abnormalities, it is possible that there could be putative genes (or multiple loci) related to trigonocephaly and, even further, to pathogenesis of the C syndrome in chromosome 3.[2,10]

We encountered a boy with the C syndrome and a de novo balanced translocation, 46,XY,t(3;18)(q13.13;q12.1).[11] By construction of a BAC/cosmid contig covering the breakpoints, we found the *CD96* (*TACTILE*) gene (GenBank accession number NM_198196) encoding a member of the immunoglobulin superfamily[12] at the 3q13.13 breakpoint (fig. 1*A*). The *CD96* gene consists of 15 exons and spans ~120 kb in the genome. Precise structural analysis around the breakpoint showed that the gene was disrupted by the translocation in exon 5, probably leading to premature termination or loss of expression of CD96 protein. There is no gene or poly-A signal in a 500-kb region telomeric to the breakpoint of chromosome 18, according to the Ensembl Genome Browser Web site. FISH analysis with use of a BAC clone, RP11-158B11, demon-

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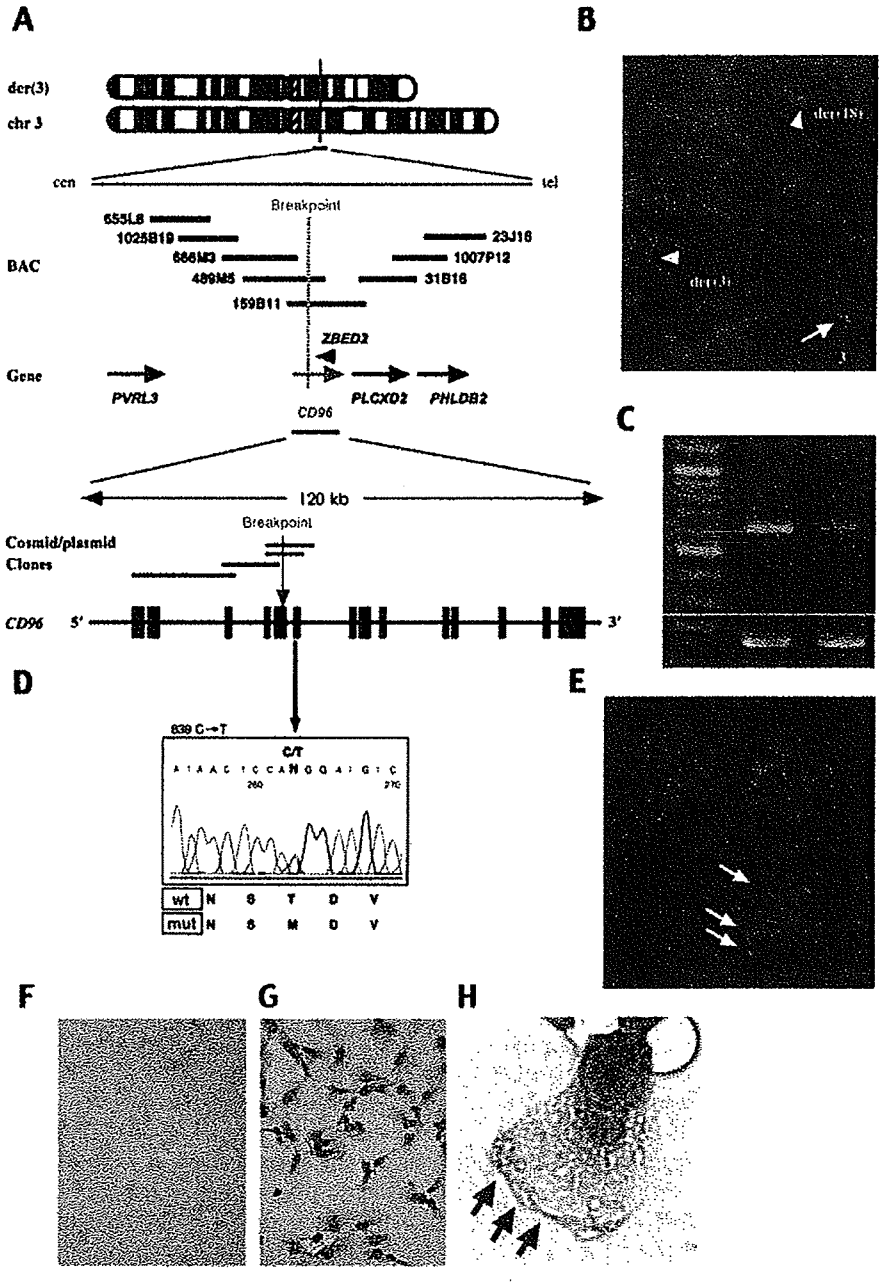

Figure 1. CD96 mutations in the C or C-like syndrome and its expression. *A*, BAC and cosmid/plasmid map spanning the 3q13.3 breakpoint of a patient with a translocation, t(3;18)(q13.13;q12.1). The red vertical line and dashed red line indicate the breakpoint. Blue bars for BACs or plasmids indicate clones covering the breakpoint (detected by FISH analysis). A red horizontal arrow indicates the *CD96* gene. A red vertical arrow indicates the breakpoint in the *CD96* gene. *B*, FISH analysis of the patient harboring the translocation. FISH signals for BAC RP11-159B11 are separated into two chromosomes, der(3) and der(18), whose signals are indicated by arrowheads. An arrow indicates signals on a normal chromosome 3. *C*, RT-PCR for *CD96* expression in the B cells of an unaffected individual and the patient with C syndrome. The left, middle, and right lanes depict a 100-bp ladder, an unaffected control, and the patient with C syndrome with translocation, respectively. The lower panel indicates expression of *GAPDH* as the control. *D*, Direct DNA-sequence analysis of *CD96* demonstrates a point mutation in a patient. *E–H*, Immunocytochemical analysis for CD96 localization in HT1080 cells with use of rabbit anti-CD96 antibody and FITC-conjugated (*E*) or HRP-conjugated (*G–H*) goat anti-rabbit IgG. *E*, Counterstained with 4′,6-diamidino-2-phenylindole (original magnification 400×). *F–H*, Counterstained with methyl green. *F*, Negative control. Original magnification was 100× (*F* and *G*) or 400× (*H*). White arrows (*E*) and black arrows (*H*) indicate regions adherent to the plastic dish.

Table 1. Comparison of Manifestations between Two of Our Patients and Frequencies of Major Findings in the C or C-like Syndromes

Clinical Finding	Presence in[a]		Frequency in	
	Patient with Translocation	Patient with Mutation	C Syndrome[2]	C-like Syndrome[4]
Trigonocephaly	+	+	23/23	13/13
Upslanting palpebral fissures	+	+	22/23	13/13
Epicanthal folds	+	−	20/22	NM
Prominent eyes	+	−	NM	13/13
Strabismus	+	+	16/22	8/8
Depressed nasal bridge	+	+	15/22	13/13
Anomalous and posteriorly angulated ears	+	+	18/21	12/13[b]
Wide alveolar ridges	+	−	10/18	4/6
High-arched palate	+	+	NM	NM
Capillary hemangioma	−	+	9/17	13/13
Redundant skin	+	−	14/20	NM
Joint contractures	−	+	7/21	13/13
Agenesis of the corpus callosum	+	+	NM	7/10
Failure to thrive	−	+	NM	11/11
Intrauterine growth retardation	−	+	NM	12/13
Seizures	−	−	5/19	5/5
Developmental retardation	±	+	18/19	9/9
Congenital heart anomalies	−	−	11/22	5/11
Clinical diagnosis	C syndrome	C-like syndrome	...	...

NOTE.—NM = not mentioned.
[a] + = present; − = absent. ± = borderline.
[b] Low-set ears.

strated split signals on the two derivative chromosomes 3 and 18 (fig. 1*B*). Semiquantitative RT-PCR analysis showed that *CD96* expression in B cells of the patient was reduced to 45.8% of the normal level (fig. 1*C*). Although one of the zinc-finger genes, *ZEBD2* (GenBank accession number NM_024508.3) exists near the breakpoint, in intron 6 of *CD96* it has the opposite direction (fig. 1*A*), and its expression was not reduced in the patient (data not shown). At the other breakpoint, 18q12.1, we could not find any genes or ESTs, according to the Genome Browser Web site (data not shown). We surveyed in this patient copy-number changes for the whole genome by the use of Human

Table 2. Sequencing Primers and PCR Conditions for the *CD96* and *ZBED2* Genes

Primer Name	Sequence (5′→3′)		T_m[a] (°C)	$MgCl_2$ (mM)	Size (bp)
	Forward	Reverse			
hCD96 ex1	CAACTGCTCTGCGTGATATC	ACCCTTAGTAATGATTTGTCCT	60	2.5	540
hCD96 ex2	CCTAAAGCAGCCAGGGAGAAA	ATGCTGAGCACCAAGCCTAAC	58	1.25	657
hCD96 ex3	GAGGACAGATGAATCCCTATAC	ATAGACTCAGAGGCTTGCCTG	60	1.8	424
hCD96 ex4	CAGACTTGCCAGTGCTGAGT	GGATGGACTAAGGTAGACTTC	60	1.8	380
hCD96 ex5	GTAAATGAATCAGTGCTTGTCGA	GTATCCAGGGAAACAGACTCC	62	2.5	429
hCD96 ex6	TCTGTATTCCCATGAAACTGTAG	TATGCAACCTGACACACCTTAC	60	1.8	367
hCD96 ex7	CATCTCTATAGGAGATAGCCCA	ACACTCCACCCCCTTGGAAG	58	1.25	472
hCD96 ex8	TTGATCATGCCATGCCTTGGC	TTTCACTGGAGTCCTACTTGTC	58	1.25	446
hCD96 ex9	GCTGCCTAGTTTCCAGGCCA	ATGGGCAAGTTAATGTGACGTG	58	1.25	485
hCD96 ex10	GGCTGTTCACTAAGATTCTTTCC	TAGTCACCGCAGAGTAACCCA	58	1.25	343
hCD96 ex11	GCCAGCTAGTGTTCCTGCATA	GTCCATGGGTGTAGTCTCAGA	60	1.8	386
hCD96 ex12	CAAGAATCCCTTCAACTCCCAC	TATATCTATCTGAGGCTGGCTTC	62	1.8	355
hCD96 ex13	CAAATCTCAGGATCCCAGCCT	TTGACCCTGACAACACCTTATC	62	1.25	499
hCD96 ex14	GCTTAGACATGCCCACCTCC	CAGCCTGACTAGGCCAATGC	62	1.25	488
hCD96 ex15	TGTGACTAACAGGCACAGGGT	GGTTAAGCTTCAGGCGTTTGG	58	1.25	467
hCD96 ex15-2	GAGAGCCAGAACTACCCAGC	CCACTCCCTACCCCCACTTT	62	1.8	372
hZBED2 15	TGTGGTTCAAATAAGCTTTTGGC	...	60	1.25	934
hZBED2 23	GTTTCGGCCAAGGGTCAGCA	...	...	...	...
hZBED2 35	ACATGATGAGGCGGGAAGACGA	...	60	1.25	657
hZBED2 43	AACAAAATGGAAGGGATGTACTG	...	...	...	...

[a] Annealing temperature.

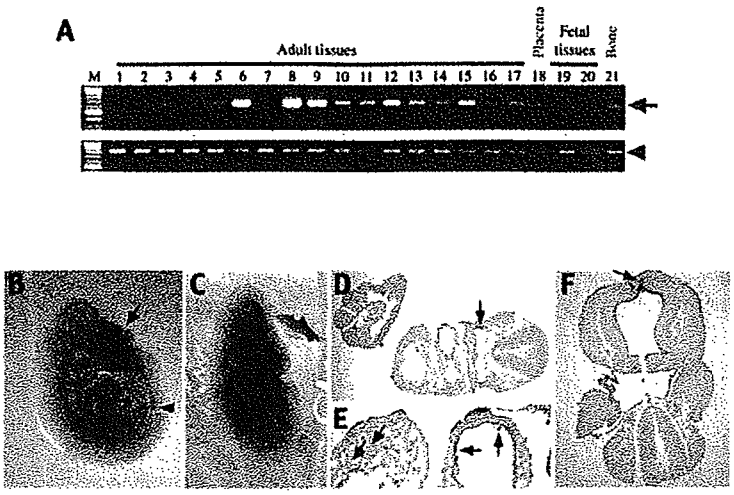

Figure 2. Expression of the *CD96* gene in fetal and adult tissues. *A*, Expression in human tissues. An arrow indicates *CD96* cDNA. An arrowhead indicates *GAPDH* cDNA as control. Lane M, size marker (100-bp ladder); 1, brain (whole); 2, cerebellum; 3, spinal cord; 4, heart; 5, kidney; 6, lung; 7, skeletal muscle; 8, spleen; 9, thymus; 10, trachea; 11, stomach; 12, small intestine; 13, colon; 14, salivary gland; 15, prostate; 16, testis; 17, uterus; 18, placenta; 19, fetal brain; 20, fetal liver; and 21, bone. Lanes 1–17 and 21, adult tissues. Lanes 19 and 20, fetal tissues. *B–F*, Whole mount *in situ* hybridization with *Cd96* antisense RNA in 10-dpc mouse embryo, showing high expression in developing forehead (*arrow in B*) and in heart and blood vessels (*arrowheads in B*). *D–F*, Horizontal sections of the embryo. *Cd96* is expressed in the pharynx (*arrow in D*); in cardiac jelly, endocardial cells, and blood cells (*arrow in E*); and in forebrain tissues (*arrow in F*). All sections are counterstained with nuclear fast red.

Mapping 50K Array Xba240 (Affymetrix). No pathogenic deletions or duplications were detected (data not shown).

We examined nine karyotypically normal Japanese patients who were given clinical diagnoses of the C or C-like syndrome. The syndromes were diagnosed by the presence of trigonocephaly and associated combinations of major clinical findings that are observed in >70% of reported patients with the C or C-like syndromes—that is, upslanting palpebral fissures, epicanthial folds, strabismus, depressed nasal root, anomalous and posteriorly angulated ears, capillary hemangioma, redundant skin, and joint contractures (table 1).[2] Two of the patients were reported as having C-like syndrome,[5,13] and the information about seven others was unpublished. First, we examined these patients for deletions or duplications by FISH analysis, using RP11-159B11 as a probe. However, no deletions were detected in any of them (data not shown). We then performed direct sequencing analysis of the candidate genes, *CD96* and *ZEBD2*. Primer pairs and PCR conditions for amplification of the candidate genes are listed in table 2. In one patient who was given a diagnosis of C-like syndrome,[5] we identified a de novo missense mutation (c.839C→T) in exon 6 of *CD96* (fig. 1*D*). The c.839C→T substitution predicts a threonine-to-methionine change (T280M) at nucleotide position 839, close to the third immunoglobulin-like domain. The threonine residue was conserved in some species—that is, chimpanzee, monkey, dog, opossum, and armadillo. The missense mutation was not found among 420 unaffected Japanese individuals.

Two patients had a homozygous 5-bp insertion (c.856-80insTTATG) in intron 6 of the *CD96* gene. They showed an ~40% reduction of *CD96* expression in their B cells, compared with the normal control level (data not shown). However, this homozygous 5-bp insertion was found in 2 of 196 normal Japanese individuals examined. No copy-number variation around this region has been registered in the Database of Genomic Variants. Therefore, it is ambiguous whether the insertion is directly associated with the syndrome. There is also a possibility that small mutations in the promoter or enhancer region of CD96 or other mutations that affect CD96 expression, albeit undetected by our analyses, might reduce the gene expression in the patients. No mutation in *ZEBD2* was found in any of the nine Japanese patients (data not shown).

We also examined 20 white patients for the *CD96* gene, 18 of whom were given clinical diagnoses of the C syndrome and 2 of whom were given diagnoses of the C-like syndrome. However, the direct sequencing analysis could not detect any apparent mutations in any exons of the *CD96* gene in these patients.

The patient having the missense mutation in *CD96* had the following relatively severe clinical manifestations: trigonocephaly, ridging of the metopic suture with narrow forehead, a small hemangioma near the nose, thin upper

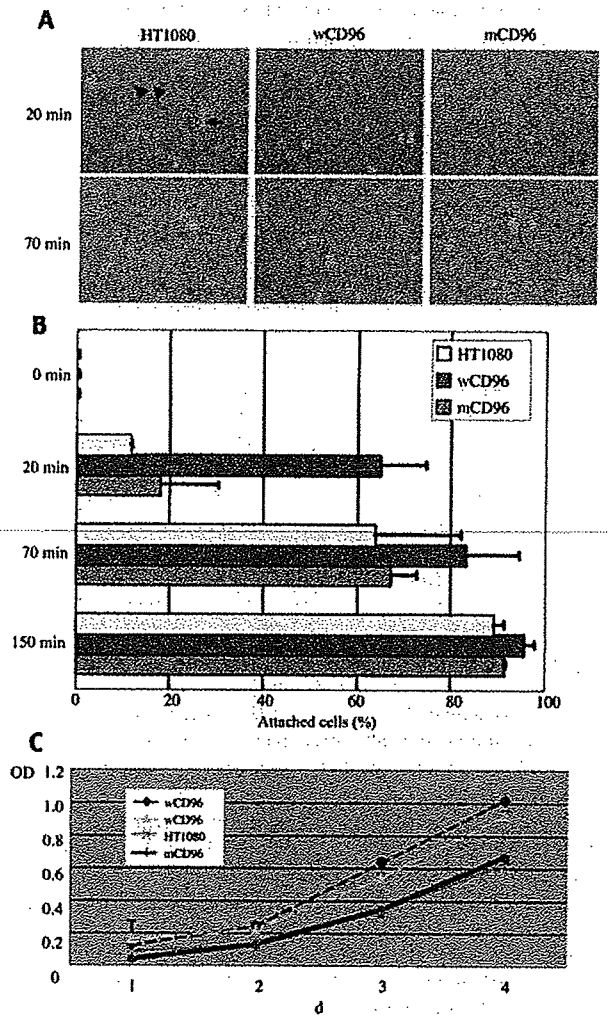

Figure 3. Functional characterization of wild-type and mutated CD96 proteins. *A* and *B*, Cell-adhesion assay for tissue-culture plates. *A*, Images captured after exposure with vibration for 20 min and 70 min. HT1080 indicates untransfected cells (control); wCD96 indicates highly expressed clone for wild-type CD96 in HT1080; and mCD96 indicates highly expressed clone for mutated CD96 in HT1080. The arrow indicates attached cell. Arrowheads indicate nonattached cells. *B*, Quantitated adhesion activity in each transformant. Attached cells and nonattached cells are counted in more than five different fields under a microscope. A total of at least 500 cells were counted for each experiment. Error bars are mean ± SD. Adhesion activities are indicated by percentages of attached cells per total cell number at 0 min, 20 min, 70 min, and 150 min after spreading cells. *C*, Cell proliferation assessed by a tetrazolium-based (MTS) assay. The ordinates show the cell number expressed as arbitrary units. Two wCD96s are clones highly expressing wild-type CD96, HT1080 is an untransfected control clone, and mCD96 is a clone expressing mutated CD96. Error bars are mean ± SD. Data shown are from three independent experiments, each performed in quadruplicate ($n = 12$).

lip, long philtrum, a high-arched palate with deep groove, low-set ears, a short neck, cryptorchidism, abnormality of the ventricular myocardium, mild optic-nerve atrophy, and hypoplasia of the corpus callosum, all of which led to the diagnosis of the C-like syndrome (table 1).[5] The patient harboring the balanced translocation had less severe manifestations—that is, trigonocephaly, a prominent metopic ridge, upslanting palpebral fissures, epicanthal folds, thick and irregular alveolar ridges, thin upper lip, long philtrum, low-set ears, redundant nuchal skin, and agenesis of the corpus callosum (table 1).[11] His phenotype satisfied the diagnosis of the C syndrome.

CD96 was identified as a human T-cell–activated antigen in long-term culture and is known to interact with the poliovirus receptor, CD155, to recognize targets for natural killer (NK) cells.[14] To determine a possible role of CD96 in the C syndrome, we investigated its expression and function in humans and mice. CD96 was found to be localized in the cytoplasm and cell-adhesion sites of the cell surface when it was expressed in HT1080 cells (fig. 1*E*–1*H*). A CD96-CFP fusion protein gave the same result when it was transiently expressed in HT1080 (data not shown). These findings support the hypothesis that CD96 may act as a cell-adhesion molecule, as do some other proteins of the immunoglobulin superfamily, such as nectin.[15] The human *CD96* gene is strongly expressed in the adult lung, spleen, and thymus and is moderately expressed in the adult spinal cord, kidney, trachea, digestive tissues, prostate, placenta, bone, and fetal brain and liver (fig. 2*A*). In 10-d-postcoitum mouse (dpc) embryos, *Cd96* is expressed in the forebrain and in a front part of the head tissues, cardiac jelly, endothelial cells, pharynx, and blood cells (fig. 2*B*–2*F*). These expression patterns are consistent with organs and tissues involved in the abnormalities of the C syndrome—that is, trigonocephaly, redundant nuchal skin, and cardiovascular abnormalities.

To analyze a potential role of CD96 in the morphological abnormalities of the C syndrome, we investigated the function of wild-type CD96 (wCD96) and mutated CD96 (mCD96 [c.839C→T]) in vitro. We constructed expression vectors for wCD96 and mCD96 using the strong CAG promoter,[16] introduced each vector into HT1080 cells, and compared the characteristics of each transformant. A cell-adhesion assay with the HT1080 cell clones expressing wCD96 showed faster attachments on tissue-culture plates compared with mock clones, even under the condition of 10% serum-containing medium (fig. 3*A* and 3*B*), whereas those expressing mCD96 showed the same adherent activity as the mock cells (fig. 3*A* and 3*B*). The result suggests that CD96 protein is involved in cell-matrix adhesion in transfected HT1080 cells, but mCD96 protein loses the activity. A tetrazolium-based (MTS) assay on the transformants, performed to determine their effect on cell growth, showed 1.5 times more growth-promoting activity of wCD96 than was shown of mCD96 in HT1080 (fig. 3*C*). Many cell-adhesion molecules belonging to the immunoglobulin super family (IgCAMs) play important roles

during embryogenesis or morphogenesis.[17] For example, mutations in the gene for PVRL1/nectin-1, a member of IgCAM, are involved in the cause of cleft lip/palate–ectodermal dysplasia syndrome (MIM #225000).[18,19]

The original report and other reports of affected sibs with the C syndrome suggested that the syndrome is inherited in an autosomal recessive fashion.[1,2] Normal chromosomes in most patients, unaffected parents with multiaffected offsprings, the equal sex ratio of affected individuals, and consanguineous matings[1,2,8] all support autosomal recessive inheritance. Meanwhile, many other patients have sporadic disease,[2] and recurrence risk may be estimated to be 10%,[8] which suggests the possibility of dominant inheritance or germline mosaicism.[2,8,10] These findings imply that the C syndrome is genetically heterogeneous, and its inheritance mode is in debate. The *CD96* aberrations found in our two patients were both in the heterozygous state without a copy-number variation in this region, which is consistent with an autosomal dominant condition. Since it is hard to assume that all reported sib cases would have originated in germline mosaicism in their respective parents, the CD96 deficiency identified in our patients cannot explain all patients with the C syndrome. However, since genetic heterogeneity is evident in the syndrome and many sporadic cases are known, our results suggest that a form of the C syndrome is caused by dysfunction of CD96. At least, the fact the mutations were found in the C and C-like syndromes may indicate that they are allelic.

A similar example is Cohen syndrome, where only ~20% of patients were found to have mutations in a causative gene, *COH1*.[20,21] The identification of a causative gene, *CD96*, may open a door to an understanding of the molecular pathology of the C syndrome.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Database of Genomic Variants, http://projects.tcag.ca/variation/
Ensembl Genome Browser, http://www.ensembl.org/index.html
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *CD96* [accession number NM_198196] and *ZBED2* [accession number NM_024508.3])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.gov/Omim/ (for C syndrome, C-like syndrome, and cleft lip/palate–ectodermal dysplasia syndrome)

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Evidence for association between a Toll-like receptor 4 gene polymorphism and moderate/severe periodontitis in the Japanese population

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Background and Objective: Chronic periodontitis is an inflammatory disease caused by bacteria in subgingival pockets. Because Toll-like receptor 2 and Toll-like receptor 4 have been shown to play an important role in the recognition of periodontal pathogens, we investigated the relevance of genetic variations in *TLR2* and *TLR4* to susceptibility to periodontitis.

Material and Methods: A total of 97 patients with chronic periodontitis and 100 control subjects were examined for mutations in *TLR2* and *TLR4*. Case-control analysis was performed using individual single nucleotide polymorphisms detected during the mutation search.

Results: The missense mutations reported previously in *TLR2* (677 Arg > Trp and 753 Arg > Gln) and in *TLR4* (299 Asp > Gly and 399 Thr > Ile) were not detected in 97 of the Japanese patients with chronic periodontitis or in 100 of the Japanese control subjects. Nine single nucleotide polymorphisms were identified in exons of *TLR2* and *TLR4*. The case-control analysis revealed that the frequency of the C/C genotype at base-pair position +3725 in *TLR4* was significantly higher in both the moderate and the severe periodontitis patient group than in the control group.

Conclusion: A genetic variation of *TLR4* might be associated with moderate and severe periodontitis in the Japanese population.

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Key words: chronic periodontitis; polymorphism; Toll-like receptor 2; Toll-like receptor 4

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Chronic periodontitis is an inflammatory disease caused by bacterial colonization in the subgingival area (1). The bacteria and their cell wall components can trigger activation of the host immune system through pattern-recognition receptors to induce inflammatory mediators, leading to the destruction of periodontal tissue. Toll-like receptor 2 and Toll-like receptor 4 are two principal pattern-recognition receptors dedicated to the recognition of bacterial cell wall components, such as lipoproteins and lipopolysaccharides (2,3). We previously demonstrated that Toll-like receptor 2 and Toll-like receptor 4 are involved in the recognition of periodontopathic bacteria, such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* (4), and that the expression of those two receptors is augmented in connective tissue subjacent to the periodontal epithelium in patients with severe periodontitis (5). These findings suggest that Toll-like receptor 2 and Toll-like

receptor 4 may be involved in the pathogenesis of periodontal diseases.

It has been reported that two missense mutations in *TLR4* (D299G and T399I) are associated with endotoxin hyporesponsiveness. The allele frequency of the D299G mutation was demonstrated to be 3–8% in European and American populations, and these mutations were associated with a blunted response of the subjects to inhaled lipopolysaccharide (6). Two missense mutations in *TLR2* (R677W and R753Q) were later identified (7,8). Although the R753Q mutation occurred in ≈3% of healthy subjects, the mutation was detected at a higher frequency (9%) in patients with gram-positive septic shock in France and was associated with a significantly reduced response to bacterial lipoprotein. The R677W mutation was detected in 10 of 45 lepromatous leprosy patients in Korea, but not found in 41 tuberculoid leprosy patients or in 45 healthy controls.

Recently, the association between these four mutations and periodontitis has been investigated. Folwaczny *et al.* found no association between chronic periodontitis and the missense mutations in *TLR2* and *TLR4* (9), and Laine *et al.* also demonstrated that the missense mutations in *TLR4* were not associated with severe periodontitis (10). On the other hand, Schröder *et al.* reported a positive association between periodontitis and the missense mutations in *TLR4* (11). They reported that patients suffering from chronic periodontitis showed a higher frequency of the missense mutations (D299G/T399I) than controls, and that the genotypes with D299G or T399I were found exclusively in patients, whereas no difference was observed for *TLR2* (11). Brett *et al.* reported, conversely, that the *TLR4* T399I minor allele was more frequent in controls than in patients with aggressive and chronic periodontitis (12). These complicated results might be explained by differences between populations.

The aim of this study was to determine whether these polymorphisms have any association with periodontitis in the Japanese population. We were unable to detect any of these four missense mutations in *TLR2/TLR4* in 197 Japanese subjects. Therefore, we attempted to identify single nucleotide polymorphisms in *TLR2* and *TLR4* in Japanese periodontitis patients, and we performed association analysis, using single nucleotide polymorphisms in *TLR2/TLR4*, to periodontitis. We show here the association between one single nucleotide polymorphism in *TLR4* and severe/moderate periodontitis in Japanese subjects.

Table 1. Classification of periodontitis patients and controls

		Classification of periodontitis		
	Controls	Mild	Moderate	Severe
Bone loss ≥ 50%	–	–	≤ 3 teeth	≥ 4 teeth
No. of subjects	100	16	65	16
Maximum PPD (mm)	2.95 ± 0.22	4.13 ± 0.50 ($p < 0.01$)	6.15 ± 0.71 ($p < 0.001$)	10.50 ± 1.46 ($p < 0.001$)

Periodontitis patients were assigned to one of three groups of disease severity on the basis of the above criteria.
p-values were calculated in comparison to the control group.
PPD, probing pocket depth.

Material and methods

Subjects

Patients with chronic periodontitis (59 women and 38 men) and healthy subjects (53 women and 47 men), who visited Nagasaki University Hospital, were enrolled in this study. All of the subjects were Japanese, resided in or around Nagasaki, and had more than 20 teeth. Individuals with malignant diseases, immunodeficiencies, pregnancy, diabetes mellitus, or who had infectious diseases, such as acquired immunedeficiency syndrome or adult T-cell leukemia, were excluded. The mean age of the patients was 60 years (range: 36–83 years) and that of the control population was 46 years (range: 25–75 years). The subjects were screened by full-mouth radiographic assessment and periodontal examinations. Subjects who had neither alveolar bone loss of > 25%, nor periodontal attachment loss of > 3 mm at any sites, were classified into the control group. Subjects who had alveolar bone loss of > 25%, or periodontal attachment loss of > 3 mm in at least at one site, were classified into the chronic periodontitis group. Periodontitis patients were further classified into three groups on the basis of the criteria of disease severity described in Table 1. To make a comparison with a group in the same age range, periodontitis patients were classified into two groups. Patients who were < 60 years of age were categorized into the younger periodontitis patient group, and patients ≥ 60 years of age were categorized into the older periodontitis patient group. The mean age of the younger periodontitis patients was 49 years and that of the older periodontitis patients was 70 years. There was no significant difference between the ages of the younger periodontitis patients and those of the control subjects. Written informed consent was obtained from all of the participants in this study.

Detection of missense mutations in *TLR2* (R677W and R753Q) and *TLR4* (D299G and T399I)

DNA was extracted from peripheral blood leukocytes by the phenol–chloroform method and harvested by ethanol precipitation. Genotyping of *TLR2* (R677W and R753Q) and *TLR4* (D299G and T399I) was accomplished with the polymerase chain reaction (PCR) and restriction enzyme digestion, following the procedures described by Schröder *et al.* (13) and Lorenz *et al.* (14), respectively.

Determination of polymorphisms/mutations in *TLR2 and TLR4*

In order to identify single nucleotide polymorphisms peculiar to the Japanese patient group, we performed direct sequencing of all the exons and introns of *TLR2* and *TLR4* (15) in 16 patients with severe periodontitis. PCR ampli-

fication was performed at various annealing temperatures using Takara ExTaq™. PCR products were sequenced using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3100 automated sequencer (Applied Biosystems). Sequencing electropherograms were aligned by ATGC software, version 3.0 (Genetyx, Tokyo, Japan), and base alterations were inspected visually.

Statistical calculation for the case-control study

As a result of single nucleotide polymorphism/mutation detection, we found five single nucleotide polymorphisms in *TLR2* and four single nucleotide polymorphisms in *TLR4*. Next, we performed genotyping of all of those single nucleotide polymorphisms in the remaining 81 patients and 100 controls. Individual single nucleotide polymorphisms were tested for Hardy–Weinberg distribution and linkage disequilibrium using SNPALYZE software (Dynacom, Yokohama, Japan). Case-control analysis was performed at individual single nucleotide polymorphisms using SNPALYZE software. The values of D′ and r^2 were calculated and referred for haplotype analysis.

Results

We failed to identify any of the reported mutations – R677W and R753Q in *TLR2* and D299G and T399I in *TLR4* – in 197 Japanese subjects, comprising 97 patients with periodontitis and 100 healthy controls.

To examine an association between *TLR2/TLR4* and periodontitis, we needed to find single nucleotide polymorphisms in *TLR2* and *TLR4*. Therefore, we decided to perform direct sequencing of *TLR2* and *TLR4* in 16 of the patients with severe periodontitis in the present study. Three point mutations in the 5′-untranslated region, and two synonymous mutations in the coding region of *TLR2*, were identified at base-pair positions −183, −148, −146, +1350 (corresponding to rs3804100) and +2343 (corresponding to rs5743709). Four point mutations were identified in the 3′-untranslated region of *TLR4* at base-pair positions +3528, +3725 (corresponding to rs11536889), +4022 and +4529 bp (Table 2). None of the mutations resulted in amino acid substitution. Seven of the nine single nucleotide polymorphisms were present at a frequency of <2% in patients and therefore would provide only a low power of association, but we performed association analysis using all of the nine single nucleotide polymorphisms. The case-control analysis revealed a significant difference between the genotype frequency of the mutation at base-pair position +3725 in *TLR4* of the patient group with that of the control group ($p = 0.043$) (Table 3). There was no significant difference between the frequencies of the other eight single nucleotide polymorphisms. Next, the genotype frequency at base-pair position +3725 in *TLR4* of the control group was compared with the genotype frequencies in the groups of patients with mild, moderate and severe periodontitis. There was no significant difference between the control group and the mild periodontitis patient group, but significant differences were found between the control group and the moderate/severe periodontitis patient groups, as

Table 2. Minor allele frequencies of single nucleotide polymorphisms in TLR2 and TLR4 genes

Gene and SNP position	SNP ID in NCBI	Nucleotide change in major/minor alleles	Minor allele frequency		HWE
			Controls	Patients	
TLR2					
−183	[a]	A/G	0	0.0053	
−148	[a]	C/T	0	0.0053	
−146	[a]	T/G	0	0.0053	$p > 0.05$
+1350	rs3804100	T/C	0.2447	0.1684	
+2343	rs5743709	G/A	0	0.0156	
TLR4					
+3528	[a]	C/G	0	0.0105	$p > 0.05$
+3725	rs11536889	G/C	0.1414	0.1959	
+4022	[a]	C/G	0	0.0053	
+4529	[a]	G/C	0.0104	0.0053	

[a]Novel single nucleotide polymorphism (SNP).
HWE, *p*-value of the Hardy–Weinberg equilibrium test in the control population. (The Hardy–Weinberg equilibrium test was performed for only two single nucleotide polymorphisms because minor allele frequencies of other single nucleotide polymorphisms were < 0.05.); ID, identity; NCBI, National Center for Biotechnology Information.

Table 3. Case-control analysis using single nucleotide polymorphisms in TLR2 and TLR4 genes

Gene and SNP position	Genotype frequency (%)						*p*-value
	Controls			Patients			
	MM	Mm	mm	MM	Mm	mm	
TLR2							
−183	93 (100)	0 (0)	0 (0)	90 (99)	1 (1)	0 (0)	0.311
−148	93 (100)	0 (0)	0 (0)	90 (99)	1 (1)	0 (0)	0.311
−146	93 (100)	0 (0)	0 (0)	90 (99)	1 (1)	0 (0)	0.311
+1350	56 (60)	30 (32)	8 (8)	66 (72)	21 (23)	5 (5)	0.214
+2343	100 (100)	0 (0)	0 (0)	93 (97)	3 (3)	0 (0)	0.082
TLR4							
+3528	97 (100)	0 (0)	0 (0)	93 (98)	2 (2)	0 (0)	0.151
+3725	73 (74)	24 (24)	2 (2)	69 (71)	18 (19)	10 (10)	0.043
+4022	92 (100)	0 (0)	0 (0)	92 (99)	1 (1)	0 (0)	0.319
+4529	94 (98)	2 (2)	0 (0)	93 (99)	1 (1)	0 (0)	0.573

SNP, single nucleotide polymorphism.

a recessive effect ($p = 0.016$ for the moderate periodontitis patient group and $p = 0.034$ for the severe periodontitis patient group) (Table 4). No significant difference was found between the control group and the mild/moderate/severe periodontitis patient groups regarding the frequencies of the other eight single nucleotide polymorphisms in *TLR2* and *TLR4*.

Because the mean age of the control group was significantly younger than that of the patient group, we classified the patients into two groups, according to their ages, to enable comparison with the group in the same age range. The frequency of the 'C/C' genotype at the +3725 base-pair position in *TLR4* in the group of younger periodontitis patients was significantly higher than that in the control group ($p = 0.022$), whereas no significant difference was found between subjects in the older periodontitis patient group and the control group (Table 5).

Discussion

The missense mutations, reported previously, in *TLR2* (R677W and R753Q) and *TLR4* (D299G and T399I), were not found in the present study (6–8). These results are consistent with the report that the D299G mutation in *TLR4* is not present in the Japanese (16) and Chinese populations (17). Because three of the four missense mutations (R753Q in *TLR2*, and D299G and T399I in *TLR4*) were reported only in European and American populations, the reason why our present results are inconsistent with previous reports might come from the differences between populations. Although R677W in *TLR2* was identified in Korean lepromatous leprosy patients, it was not detected in 286 Indian lepromatous leprosy patients (18). It was suggested that the R677W mutation might come from the variation in the duplicated region with 93% homology to *TLR2* exon 3 located at ≈23 kb 5′-position to the functional *TLR2* gene (18). Because the primers we used in this study were designed specifically for the functional *TLR2* gene, our results are definitive. Although there are conflicting results in the literature regarding the association between those four missense mutations and the susceptibility to periodontitis (9–12), it is difficult to use those four missense mutations to confirm the association between periodontitis and *TLR2/TLR4* in the Japanese population because those missense mutations are very rare in Japanese subjects.

We searched for single nucleotide polymorphisms around exons in *TLR2/TLR4* because it is possible that other single nucleotide polymorphisms, previously reported, are associated with periodontitis in Japanese subjects. We found nine single nucleotide polymorphisms in the exons of *TLR2* and *TLR4* in the present study; however, none resulted in amino acid substitution. A missense mutation(s) in *TLR2/TLR4* would probably not be found as a common variation in Japanese periodontitis patients. It is possible that periodontitis is based on many rare variants, although we did not perform a mutation search in *TLR2/TLR4* in all of the patients. Smirnova *et al.* reported that 11 rare missense mutations in *TLR4* were found in 197 meningococcal patients, but that only one rare missense mutation was identified in 127 controls in the UK (19). This is an example that is consistent with the hypothesis that many rare variants are related to common diseases.

We found that the *TLR4* +3725G > C mutation was associated with the whole periodontitis group, and a significant association was also found between the control group and the moderate/severe periodontitis patient groups. Although the mean age of the control group was lower than that of the patient group, the 'C/C' genotype was observed more frequently in the younger periodontitis patient group than in the control group. The positive results from the whole case-control study, and the comparison between the age-matched groups, strongly suggest that the *TLR4* +3725G > C mutation is associated with periodontitis. We did not perform a haplotype association study because the single nucleotide polymorphisms used in this study showed no evidence of linkage disequilibrium (data not shown) with each other. In our next research step, we need to perform a mutation search for other base changes within the genomic region, including *TLR4*. Such a study will uncover the single nucleotide polymorphisms in

Table 4. TLR4 +3725 (rs11536889) genotype frequencies in periodontitis patients (mild, moderate, severe) and control subjects

SNP ID	Subjects	Genotype GG (%)	GC (%)	CC (%)	*p*-value
rs11536889	Mild	9 (63)	6 (32)	1 (5)	*P1* = 0.325 *P2* = 0.151
	Moderate	47 (71)	11 (18)	7 (11)	*P1* = 0.016 *P2* = 0.840
	Severe	13 (81)	1 (6)	2 (13)	*P1* = 0.034 *P2* = 0.521
	Control	73 (74)	24 (24)	2 (2)	

ID, identity; SNP, single nucleotide polymorphism.
P1: *p*-value considered as the C allele having a recessive effect (GG + GC vs. CC).
P2: *p*-value considered as the C allele having a dominant effect (GG vs. GC + CC).

Table 5. TLR4 +3725 (rs11536889) genotype frequencies in the younger periodontitis patient group (< 60 years of age) and in the older periodontitis patient group (≥ 60 years of age)

Age-group	Genotype GG (%)	GC (%)	CC (%)	*p*-value
Younger	32 (70)	8 (17)	6 (13)	0.022
Older	37 (72)	10 (20)	4 (8)	0.204

p-values were calculated in comparison to the control group.

linkage disequilibrium with +3725G > C of the *TLR4* gene or the disease-associated haplotype within the *TLR4* gene. Considering that the progression of periodontitis is affected by multiple factors, such as oral hygiene and the deposition of calculus, a genetic influence may not be sufficient to distinguish the mild periodontitis group from the control group. Age is also known to be a putative risk factor for periodontitis (20), and older patients, with a relatively low-genetic background of mutations, might be suffering from periodontitis. Those factors may account for the lack of statistical difference between the control group and the mild periodontitis group, and between the control group and the older periodontitis group.

Because the +3725G > C mutation is located in the 3′-untranslated region of *TLR4*, it does not have any direct influence on the conformation of the Toll-like receptor 4 protein molecule, according to our present biological knowledge. However, because single nucleotide polymorphisms in introns and/or untranslated regions may influence transcription and/or translation (21–24), the +3725G > C mutation might have a direct effect on mRNA stability or translation efficiency. Antisense transcripts might be important for regulating *TLR4* transcription. The reported disease-associated single nucleotide polymorphisms or haplotypes are not always found in coding regions in 'common diseases' (25,26). The functional assay of disease association with single nucleotide polymorphisms in introns is the next point requiring investigation. In view of the importance of the Toll-like receptor 4 in the pathogenesis of periodontal diseases, the biological significance of genetic variation, including transcription efficiency of the mutated gene, needs to be elucidated.

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Prenatal ultrasonographic findings may be useful in predicting the prognosis of trisomy 18

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Objective This study was conducted to determine whether specific ultrasonographic findings in the prenatal period were associated with survival of children affected with trisomy 18.

Method Between October 1987 and July 2004, we collected 24 cases with trisomy 18. We investigated the relationship of prenatal ultrasonographic findings, gestational age at delivery, the Apgar score, the mode of delivery, and the neonatal treatment strategies, with survival time.

Results 17 of the 24 neonates died within 1 month after birth (Group 1), 5 died within 12 months (Group 2), and 2 survived for more than 1 year (Group 3). Severe polyhydramnios was more frequent in group 1. In groups 2 and 3, the fluid in fetal stomach was visible in all cases. Severe cardiac anomalies were more frequent in group 1. In groups 2 and 3, all neonates were girl.

Conclusion Prenatal ultrasonographic findings associated with survival less than 1 month included severe polyhydramnios, absence of the fluid in the stomach, severe cardiac anomaly and male sex. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: trisomy 18; survival; ultrasonography

INTRODUCTION

Trisomy 18 is the second most frequent chromosomal abnormality in live-borns following trisomy 21, with an incidence reported to be 1/7000 neonates (Goldstein and Nielsen, 1988). This disorder is definitively diagnosed by karyotype analysis; however, recently, prenatal ultrasonographic findings have suggested this disorder in many cases (Nyberg *et al.*, 1993; Nyberg and Souter, 2001; Tongsong *et al.*, 2002). Although the prognosis of trisomy 18 is poor, some patients have been reported as surviving for a long period (Kelly *et al.*, 2002; Slavotinek *et al.*, 2003). The prognostic factors hitherto reported include gender difference (boys<girls), the presence or absence of cardiac anomalies, and neonatal treatment strategies (Sonja *et al.*, 2003; Taylor, 1968; Root and Carey, 1994); however, these factors remain to be clarified in many respects. In particular, information on the prognosis of this disorder is important for counseling with patients' parents. Prenatal ultrasonographic findings associated with this disorder include polyhydramnios, intrauterine growth restriction, various malformations such as cardiac anomalies, rachischisis, cerebellar hypoplasia, micrognathia, umbilical hernia, clenched hands/wrists, radial defect, clubfeet, cystic hygroma colli, and single umbilical artery; however, they vary considerably among patients (Nyberg *et al.*, 1993; Nyberg and Souter, 2001; Tongsong *et al.*, 2002).

In this study, we investigated the relation between fetal ultrasonographic findings and survival to identify specific ultrasonographic findings strongly associated with the outcome of infants affected with trisomy 18.

METHODS

Of 29 fetuses diagnosed as having trisomy 18 in Nagasaki University Hospital and Sasebo City General Hospital between October 1987 and July 2004, the subjects were 24 fetuses excluding two spontaneous abortions, two artificial abortions, and one fetus of which the details were unclear.

In the first trimester, gestational age was determined from the final menstruation or fetal crown–rump length. In all subjects, ultrasonographic findings suggested fetal anomalies. After genetic counseling, amniocentesis was performed based on the parents' wishes. Trisomy 18 was diagnosed by a chromosome test using amniotic fluid and/or neonatal blood. There was no mosaicism. In fetuses in which a prenatal diagnosis was made, additional genetic counseling was conducted with the parents, and the mode of delivery was selected.

We investigated the correlation between ultrasonographic findings in fetuses with trisomy 18 and their survival after birth. Ultrasonographic findings included amniotic fluid volume, the presence or absence of fluid in the fetal stomach, the presence or absence of cardiac anomalies, the anteroposterior diameter of the cisterna magna, and the grade of intrauterine fetal growth restriction. Other factors that could have affected survival, such as the gestational age at delivery, Apgar score, and mode of delivery, were described. Neonatal treatment strategies were also involved, e.g. whether

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the neonate was admitted to the neonatal intensive care unit (NICU) and whether surgery for malformations was performed. Amniotic fluid volume was estimated by the amniotic fluid pocket: deepest pocket free of umbilical cord or fetal parts in the anteroposterior plane of the uterus. An amniotic fluid pocket of 120 mm or more was regarded as severe polyhydramnios. The grade of intrauterine growth restriction was compared using deviations from the standard fetal body weight in Japan. An Apgar score of less than 3 after 1 min was regarded as low.

RESULTS

Concerning fetal survival, 17 of the 24 neonates died within 1 month after birth (Table 1). Of the other 7 neonates, 5 died within 12 months, whereas 2 survived for more than 12 months (patient 23: more than 5 years, patient 24: more than 1 year). The above 17 neonates (patients 1–17) were assigned to group 1 (17/24, 70.8%), 5 (patients 18–22) were assigned to group 2 (5/24, 20.8%), and 2 (patients 23 and 24) were assigned to group 3 (2/24, 8%). Severe polyhydramnios (amniotic fluid pocket: 120 mm or more) was observed in 12 of the 17 neonates in group 1, and in 1 of the 2 neonates in group 3. Amnioreduction had been performed due to polyhydramnios during pregnancy in 8 neonates in group 1, and in 1 neonate in group 3 (Tables 2 and 3). As cardiac anomalies, ventricular septal defect, atrial septal defect, hypoplastic left heart syndrome, and single atrium/single ventricle were noted (Table 2). There were no significant differences in the incidence of cardiac anomalies among the 3 groups (Table 3); however, severe cardiac anomalies, such as hypoplastic left heart syndrome, were more frequent in group 1 (Table 2).

There were no significant differences in the anteroposterior diameter of the cisterna magna among the 3 groups (Table 3).

In groups 2 and 3, ultrasonographic findings showed fluid in the stomach in all neonates; however, fluid in the stomach was not observed in group 1, with significant differences.

The grade of intrauterine growth restriction was -2.3 ± 0.7 in group 1, -2.9 ± 0.8 in group 2, and -2.6 ± 0.9 in group 3; there were no significant differences among the 3 groups. Fourteen of the 17 neonates in group 1 showed low Apgar scores (less than 3 after 1 min), comprising a higher percentage. In group 1, 9 of the 17 neonates were boys. In groups 2 and 3, all neonates were girls. Premature birth accounted for approximately 50% of the neonates in groups 1 and 2. In group 3, there were no premature births. Concerning the mode of delivery, cesarean section was selected for 1 of the 17 neonates in group 1, and in 4 of the 5 neonates in group 2. In the 2 neonates in group 3, transvaginal delivery was selected. In group 1, 3 neonates were admitted to the NICU (cases 1–3), and case 2 received mechanical ventilation. In group 2, all neonates were admitted to the NICU, and cases 19 and 20 received mechanical ventilation. The 2 neonates in group 3 were not admitted to the NICU (Table 3). No subject underwent surgery in the neonatal period.

The cause of death in neonates/infants managed in the NICU was either respiratory failure or heart failure. Case 2 with a single atrium/single ventricle died of heart failure. Case 3 died of progressive respiratory failure. Cases 18, 20 and 21, who had large VSD, died of pulmonary hypertension and heart failure. Case 19 died of infection of the respiratory tract. Case 22 died of sudden cardiac arrest. The neonates with diaphragmatic hernia (cases 13 and 14) died of respiratory failure. We did not know the exact cause of death in patients who were not managed in the NICU.

DISCUSSION

The prognosis of trisomy 18 is generally poor, and many patients die during the first days after birth; however, some patients have been reported as surviving for a long period (Kelly *et al.*, 2002; Slavotinek *et al.*, 2003). According to previous studies regarding the outcome of neonates with trisomy 18, 38.6% of 114 neonates survived for 1 month, and 8.4% survived for 1 year. The mean survival was 14.5 days (Sonja *et al.*, 2003).

We examined the association of fetal ultrasonographic findings (the amniotic pocket, fluid in the fetal stomach, presence or absence of cardiac anomalies, anteroposterior diameter of the cisterna magna, and the grade of intrauterine growth restriction), gestational age at delivery, Apgar score, mode of delivery, and admission to the NICU, with survival after birth. In particular, neonates survived more than 1 month (group 2 and group 3) when fluid in the stomach was detected and without severe polyhydramnios. The absence of fluid in the stomach and polyhydramnios are often associated with esophageal atresia and/or functional swallowing disorder. Both could result in poor nutritional status in the neonatal period. In addition, preterm birth and resulting neonatal prematurity are common in cases of severe polyhydramnios that causes marked uterine overdistension. Indeed, in the present study, the average gestational age at delivery in group 1 was earlier than in groups 2 and 3. A recent report on 161 neonates affected by trisomy 18 showed similar results: esophageal atresia was associated with shorter survival and prematurity (Niedrist *et al.*, 2006). Palliative surgery for gastrointestinal malformation seems to be rejected for the majority of trisomy 18 cases, probably because of the well-known lethality of the disorder; however, it might be of value to investigate whether active treatment strategies lengthen the survival time.

In our study, we did not find an apparent relation between the presence or absence of cardiac anomalies and the prognosis of trisomy 18, differing from another report on 27 cases (Taylor, 1968). Although this lack of consistency could be due to the small sample size, another report with a larger sample size (Niedrist *et al.*, 2006) suggested that the presence of VSD, the commonest heart anomaly seen in trisomy 18, does not influence

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Table 1—Details of 24 diagnostic cases of trisomy 18

Case no.	Maternal age	Outcome	Gestational age at delivery (weeks)	Birth weight (g)	Apgar score (1 min/5 min)	NICU	Mechanical ventilation	Cause of death
1	31	Died within 23 days	41	2250	2/5	+	−	Heart failure
2	23	Died within 16 days	37	1796	NA	+	+	Heart failure
3	40	Died within 22 days	33	1234	8/8	+	−	Respiratory failure
4	27	Died within 15 days	37	1614	NA	−	−	NA
5	33	Died within 4 days	35	1100	2/2	−	−	NA
6	28	Died within 2 hours	33	1132	2/NA	−	−	NA
7	32	Died soon after delivery	32	1234	NA	−	−	NA
8	29	Died within 1 day	30	1230	NA	−	−	NA
9	27	Died within 4 h	33	1140	1/4	−	−	NA
10	31	Died within 1 h	37	1800	3/1	−	−	NA
11	31	Died soon after delivery	31	1350	1/1	−	−	NA
12	44	Died within 20 h	36	1660	1/1	−	−	NA
13	39	Died within 2 h	37	1960	4/1	−	−	Respiratory failure
14	21	Died within 1 h	36	1840	6/4	−	−	Respiratory failure
15	38	IUFD in labor	38	1850	0/0	−	−	NA
16	38	Died within 10 h	37	1420	2/2	−	−	NA
17	27	IUFD in labor	37	1600	0/0	−	−	NA
18	31	Died within 1 month	38	1872	8/9	+	−	PH
19	38	Died within 11 months	37	1222	1/NA	+	+	Infection of the respiratory tract
20	30	Died within 4 months	34	1318	5/7	+	+	PH
21	30	Died within 4 months	34	1378	6/7	+	−	PH
22	36	Died within 5 months	41	2052	3/6	+	−	Sudden cardiac arrest
23	19	Survived (more than 5 years)	39	1970	7/7	−	−	
24	28	Survived (more than 1 year)	41	2510	7/8	−	−	

IUFD, intra uterine fetal death; NICU, neonatal intensive care unit, +: admitted to the NICU; mechanical ventilation, +: received mechanical ventilation; PH, pulmonary hypertension; NA, not available.

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