**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.

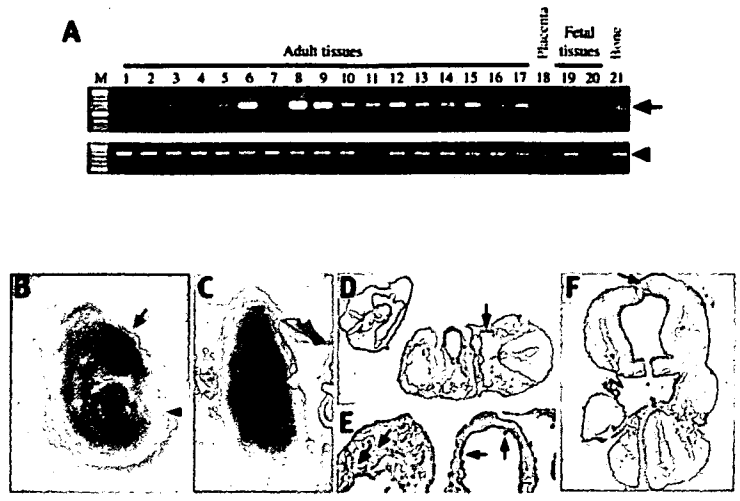


**Figure 2.** Expression of the *CD96* gene in fetal and adult tissues. *A*, Expression in human tissues. An arrow indicates *CD96* cDNA. An arrowhead indicates *GAPDH* cDNA as control. Lane M, size marker (100-bp ladder); 1, brain (whole); 2, cerebellum; 3, spinal cord; 4, heart; 5, kidney; 6, lung; 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

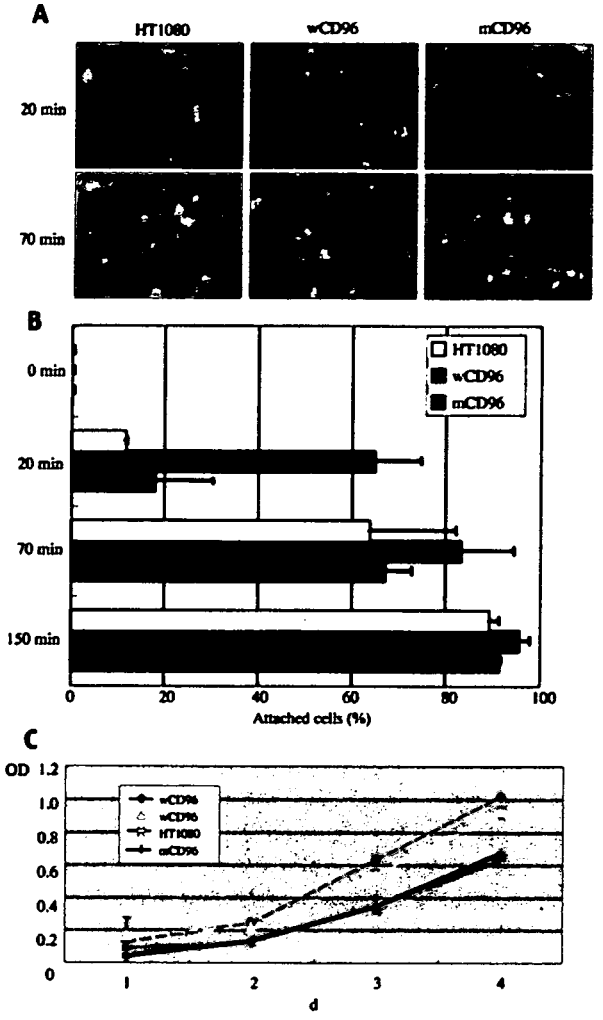


**Figure 3.** Functional characterization of wild-type and mutated CD96 proteins. *A* and *B*, Cell-adhesion assay for tissue-culture plates. *A*, Images captured after exposure with vibration for 20 min and 70 min. HT1080 indicates untransfected cells (control); wCD96 indicates highly expressed clone 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.

## Acknowledgments

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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)

## References

1. Opitz JM, Johnson RC, McCreadie SR, Smith DW (1969) The C syndrome of multiple congenital anomalies. Birth Defects 5:161–166
2. Gorlin RJ, Cohen MM Jr, Hennekam RCM (2001) Syndromes of the head and neck, 4th ed. Oxford University Press, New York, pp 1145–1147
3. Bohring A, Silengo M, Lerone M, Superneau DW, Spaich C, Braddock SR, Poss A, Opitz JM (1999) Severe end of Opitz trigonocephaly (C) syndrome or new syndrome? Am J Med Genet 85:438–446
4. Bohring A, Oudesluijs GG, Grange DK, Zampino G, Thierry P (2006) New cases of Bohring-Opitz syndrome, update, and critical review of the literature. Am J Med Genet A 140:1257–1263
5. Osaki M, Makita Y, Miura J, Abe N, Noguchi S, Miyamoto A (2006) A Japanese boy with apparent Bohring-Opitz or "C-like" syndrome. Am J Med Genet A 140:897–899
6. Schwyzer U, Binkert F, Caflisch U, Baumgartner B, Schinzel A (1987) Terminal deletion of the short arm of chromosome 3, del(3pter-p25): a recognizable syndrome. Helv Paediatr Acta 42:309–311
7. McGaughran J, Aftimos S, Oei P (2000) Trisomy of 3pter in a patient with apparent C (trigonocephaly) syndrome. Am J Med Genet 94:311–315
8. Sargent C, Burn J, Baraitser M, Pembrey ME (1985) Trigonocephaly and the Opitz C syndrome. J Med Genet 22:39–45
9. Preus M, Vekemans M, Kaplan P (1986) Diagnosis of chromosome 3 duplication q23→qter, deletion p25→pter in a patient with the C (trigonocephaly) syndrome. Am J Med Genet 23:935–943
10. Opitz JM, Putnam AR, Comstock JM, Chin S, Byrne JL, Kennedy A, Frikke MJ, Bernard C, Albrecht S, Der Kaloustian V, et al (2006) Mortality and pathological findings in C (Opitz trigonocephaly) syndrome. Fetal Pediatr Pathol 25:211–231
11. Chinen Y, Kaname T, Yanagi K, Naritomi K, Ohta T (2006) Opitz trigonocephaly C syndrome in a boy with a *de novo* balanced reciprocal translocation t(3;18)(q13.13;q12.1). Am J Med Genet A 140:1655–1657
12. Wang PL, O'Farrell S, Clayberger C, Krensky AM (1992) Identification and molecular cloning of TACTILE: a novel human T cell activation antigen that is a member of the Ig gene superfamily. J Immunol 148:2600–2608
13. Nakane T, Kubota T, Fukushima Y, Hata Y, Ishii J, Komiyama A (2000) Opitz trigonocephaly (C)-like syndrome, or Bohring-Opitz syndrome: another example. Am J Med Genet 92:361–362
14. Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M (2004) CD96 (Tactile) promotes NK cell-target cell adhesion by interacting with the poliovius receptor (CD155). J Immunol 172:3994–3998
15. Sakisaka T, Takai Y (2004) Biology and pathology of nectins and nectin-like molecules. Curr Opin Cell Biol 16:513–521
16. Kaname T, Huxley C (2001) Simple and efficient vectors for retrofitting BACs and PACs with mammalian neoR and EGFP marker genes. Gene 266:147–153
17. Krauss RS, Cole F, Gaio U, Takaesu G, Zhang W, Kang JS (2005) Close encounters: regulation of vertebrate skeletal myogenesis by cell-cell contact. J Cell Sci 118:2355–2362
18. Suzuki K, Hu D, Bustos T, Zlotogora J, Richieri-Costa A, Helms

JA, Spritz RA (2000) Mutation of PVRL1, endoding a cell-cell adhesion molecule/herpesvirus receptor, in cleft lip/palate-ectodermal dysplasia. Nat Genet 25:427–430
19. Sozen MA, Suzuki K, Tolarova M, Bustos T, Fernandez Iglesias JE, Spritz RA (2001) Mutation of PVRL1 is associated with sporadic, non-syndromic cleft lip/palate in northern Venezuela. Nat Genet 29:141–142
20. Kolehmainen J, Wilkinson R, Lehesjoki AE, Chandler K, Kivitie-Kallio S, Clayton-Smith J, Traskelin AL, Waris L, Saarinen A, Khan J, et al (2004) Delineation of Cohen syndrome following a large-scale genotype-phenotype screen. Am J Hum Genet 75:122–127
21. Mochida GH, Rajab A, Eyaid W, Lu A, Al-Nouri D, Kosaki K, Noruzinia M, Sarda P, Ishihara J, Bodell A, et al (2004) Broader geographical spectrum of Cohen syndrome due to COH1 mutations. J Med Genet 41:e87

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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

| | Controls | Classification of periodontitis | | |
|---|---|---|---|---|
| | | 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 (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Controls | | | Patients | | | |
| | MM | Mm | mm | MM | Mm | mm | *p*-value |
| *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) | *P*1 = 0.325<br>*P*2 = 0.151 |
| | Moderate | 47 (71) | 11 (18) | 7 (11) | *P*1 = 0.016<br>*P*2 = 0.840 |
| | Severe | 13 (81) | 1 (6) | 2 (13) | *P*1 = 0.034<br>*P*2 = 0.521 |
| | Control | 73 (74) | 24 (24) | 2 (2) | |

ID, identity; SNP, single nucleotide polymorphism.
*P*1: *p*-value considered as the C allele having a recessive effect (GG + GC vs. CC).
*P*2: *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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## References

1. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25**:134–144.
2. Lien E, Sellati TJ, Yoshimura A *et al.* Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J Biol Chem* 1999;**274**:33419–33425.
3. Hoshino K, Takeuchi O, Kawai T *et al.* Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J Immunol* 1999; **162**:3749–3752.
4. Yoshimura A, Kaneko T, Kato Y, Golenbock DT, Hara Y. Lipopolysaccharides from periodontopathic bacteria *Porphyromonas gingivalis* and *Capnocytophaga ochracea* are antagonists for human Toll-like receptor 4. *Infect Immun* 2002;**70**:218–225.
5. Mori Y, Yoshimura A, Ukai T, Lien E, Espevik T, Hara Y. Immunohistochemical localization of Toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. *Oral Microbiol Immunol* 2003;**18**:54–58.
6. Arbour NC, Lorenz E, Schutte BC *et al.* *TLR4* mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000;**25**:187–191.
7. Lorenz E, Mira JP, Cornish KL, Arbour NC, Schwartz DA. A novel polymorphism in the Toll-like receptor 2 gene and its potential association with staphylococcal infection. *Infect Immun* 2000;**68**:6398–6401.
8. Kang TJ, Chae GT. Detection of Toll-like receptor 2 (TLR2) mutation in the lepromatous leprosy patients. *FEMS Immunol Med Microbiol* 2001;**31**:53–58.
9. Folwaczny M, Glas J, Torok HP, Limbersky O, Folwaczny C. Toll-like receptor (TLR) 2 and 4 mutations in periodontal disease. *Clin Exp Immunol* 2004;**135**:330–335.
10. Laine ML, Morre SA, Murillo LS, van Winkelhoff AJ, Pena AS. *CD14* and *TLR4* gene polymorphisms in adult periodontitis. *J Dent Res* 2005;**84**:1042–1046.
11. Schröder NW, Meister D, Wolff V *et al.* Chronic periodontal disease is associated with single-nucleotide polymorphisms of the human *TLR-4* gene. *Genes Immun* 2005;**6**:448–451.
12. Brett PM, Zygogianni P, Griffiths GS *et al.* Functional gene polymorphisms in aggressive and chronic periodontitis. *J Dent Res* 2005;**84**:1149–1153.
13. Schröder NW, Hermann C, Hamann L, Gobel UB, Hartung T, Schumann RR. High frequency of polymorphism Arg753Gln of the Toll-like receptor-2 gene detected by a novel allele-specific PCR. *J Mol Med* 2003;**81**:368–372.
14. Lorenz E, Hallman M, Marttila R, Haataja R, Schwartz DA. Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population. *Pediatr Res* 2002;**52**:373–376.
15. Ichikawa E, Watanabe A, Nakano Y *et al.* *PAX9* and *TGFB3* are linked to susceptibility to nonsyndromic cleft lip with or without cleft palate in the Japanese: population-based and family-based candidate gene analyses. *J Hum Genet* 2006;**51**:38–46.
16. Okayama N, Fujimura K, Suehiro Y *et al.* Simple genotype analysis of the Asp299Gly polymorphism of the *Toll-like receptor-4* gene that is associated with lipopolysaccharide hyporesponsiveness. *J Clin Lab Anal* 2002;**16**:56–58.
17. Hang J, Zhou W, Zhang H *et al.* *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms are very rare in the Chinese population. *J Endotoxin Res* 2004;**10**:238–240.
18. Malhotra D, Relhan V, Reddy BS, Bamezai R. TLR2 Arg677Trp polymorphism in leprosy: revisited. *Hum Genet* 2005;**116**:413–415.
19. Smirnova I, Mann N, Dols A *et al.* Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility. *Proc Natl Acad Sci USA* 2003;**100**:6075–6080.
20. Tonetti MS, Claffey N. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *J Clin Periodontol* 2005;**32**:210–213.
21. Bream JH, Carrington M, O'Toole S *et al.* Polymorphisms of the human *IFNG* gene noncoding regions. *Immunogenetics* 2000;**51**:50–58.
22. Borrmann L, Wilkening S, Bullerdiek J. The expression of *HMGA* genes is regulated by their 3′UTR. *Oncogene* 2001; **20**:4537–4541.
23. Rousseau P, Le Discorde M, Mouillot G, Marcou C, Carosella ED, Moreau P. The 14 bp deletion-insertion polymorphism in the 3′ UT region of the HLA-G gene influences HLA-G mRNA stability. *Hum Immunol* 2003;**64**:1005–1010.
24. Hesketh J. 3′-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. *Biochem Soc Trans* 2004;**32**:990–993.
25. Curran JE, Jowett JB, Elliott KS *et al.* Genetic variation in selenoprotein S influences inflammatory response. *Nat Genet* 2005;**37**:1234–1241.
26. Grant SF, Thorleifsson G, Reynisdottir I *et al.* Variant of transcription factor 7-like 2 (*TCF7L2*) gene confers risk of type 2 diabetes. *Nat Genet* 2006;**38**:320–323.

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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 $-2.3 \pm 0.7$ in group 1, $-2.9 \pm 0.8$ in group 2, and $-2.6 \pm 0.9$ 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.

Table 2—Prenatal ultrasonographic findings

| Case no. | Cardiac anomaly | Fluid in the fetal stomach | AFV (mm) | Amnioreduction | Cisterna magna (mm) | Other findings |
|---|---|---|---|---|---|---|
| 1 | Cardiomegaly | + | 115 | No | 26 | Single umbilical artery, overlapping fingers |
| 2 | SA/SV | + | 130 | No | 25 | Single umbilical artery, overlapping fingers |
| 3 | VSD | + | 74 | No | 13 | Absent |
| 4 | NA | + | 164 | No | 20 | Overlapping fingers, strawberry-shaped skull |
| 5 | HLHS | − | 142 | Yes | 23 | Single umbilical artery, overlapping fingers, esophageal atresia, mandibular hypoplasia |
| 6 | HLHS | − | 107 | No | 15 | Overlapping fingers, strawberry-shaped skull |
| 7 | NA | − | 140 | Yes | 12 | Overlapping fingers, esophageal atresia, hydronephrosis |
| 8 | HLHS | − | 149 | No | 8.5 | Single umbilical artery |
| 9 | HLHS | − | 130 | No | 20 | Overlapping fingers, mandibular hypoplasia, ocular anomalies |
| 10 | SA/SV | − | 160 | Yes | 15 | Absent |
| 11 | Cardiomegaly | − | 164 | Yes | 18 | Single umbilical artery, umbilical hernia, esophageal atresia |
| 12 | Cardiomegaly | − | 114 | No | 13 | Umbilical hernia, clubfeet, radial ray anomalies, rocker-bottom feet, cystic hygroma colli |
| 13 | Cardiomegaly | − | 96 | No | 15 | Overlapping fingers, diaphragmatic hernia, radial ray anomalies, rocker-bottom feet |
| 14 | NA | − | 178 | Yes | 16 | Overlapping fingers, diaphragmatic hernia, esophageal atresia, clubfeet |
| 15 | VSD | + | 152 | Yes | NA | Overlapping fingers, hydronephrosis, clubfeet, ocular anomalies, cleft lip |
| 16 | Absent | + | 135 | Yes | 6 | Single umbilical artery, overlapping fingers, hydronephrosis |
| 17 | NA | − | 130 | Yes | 12 | Absent |
| 18 | VSD | + | 69 | No | 25 | Absent |
| 19 | PDA, VSD | + | 69 | No | 18 | Overlapping fingers |
| 20 | VSD | + | NA | No | NA | Absent |
| 21 | PDA, ASD, VSD | + | 85 | No | NA | Absent |
| 22 | PDA, ASD | + | 106 | No | 9 | Overlapping fingers, rocker-bottom feet |
| 23 | Absent | + | 80 | No | 20 | Absent |
| 24 | VSD | + | 126 | Yes | 5 | Single umbilical artery |

SA/SV, single atrium/single ventricle; VSD, ventricular septal defect; HLHS, hypoplastic left heart syndrome; PDA, patent ductus arteriosus; ASD, atrial septal defect; AFV, amniotic fluid volume.




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Table 3—Prenatal ultrasonographic and neonatal findings of the 3 groups

| | Group 1 ($n$ = 17) | Group 2 ($n$ = 5) | Group 3 ($n$ = 2) |
|---|---|---|---|
| Sex (male) | 9 (53%) | 0 | 0 |
| Severe polyhydramnios[a] | 12 (70%) | 0 | 1 (50%) |
| Amnioreduction | 8 (66%) | 0 | 1 (50%) |
| Cardiac anomaly | 12 (70%) | 5 (100%) | 1 (50%) |
| Stomach not visualized | 11 (64%) | 0 | 0 |
| Cisterna magna (mm)[b] | 16 | 17 | 12 |
| Grade of IUGR | $-2.3 \pm 0.7$ | $-2.9 \pm 0.8$ | $-2.6 \pm 0.9$ |
| Premature birth | 8 (47%) | 2 (40%) | 0 |
| Cesarean section | 1 (5.9%) | 4 (80%) | 0 |
| Low Apgar score[c] | 14 (82%) | 2 (40%) | 0 |
| NICU | 3 (17%) | 5 (100%) | 0 |

[a] amniotic fluid pocket >120 mm.
[b] average.
[c] less than 3 after 1 min.
Data are expressed as the number (%) or mean ±SD; IUGR, intrauterine growth restriction.

prognosis. We should probably take into account the severity of cardiac anomalies. Indeed, in our study, 4 cases of hypoplastic left heart syndrome died within 4 days after birth.

Fifteen of the 24 children delivered with trisomy 18 were girls, and no boys survived for longer than 1 month. Previous studies consistently showed more girls born alive and longer survival of girls, for unknown reasons (Root and Carey, 1994).

A recent report suggested that neonatal intensive treatment, including cesarean section, resuscitation by intubation and surgery, improved the survival of neonates affected by trisomy 18: survival rates at ages 1 week, 1 month, and 1 year were 88, 83, and 25%, respectively (Kosho *et al.*, 2006). All cases were prenatally diagnosed, as were those in our study. In our series, the 5 neonates in group 2 underwent active management in the NICU, survived more than 1 month but died within 5 months. It is likely that intensive treatment might improve the relatively short-term prognosis. Future work should further examine the effect as well as the indication of active management on survival for a longer period.

Concerning the mode of delivery, cesarean section was selected for 4 of the 5 neonates in group 2, and transvaginal delivery for the 2 neonates in group 3. These results show that the mode of delivery *per se*, has no impact on the prognosis of trisomy 18; however, cesarean section performed for obstetrical indications such as fetal malpresentation, dystocia, or fetal distress should improve at the least short-term prognosis.

## CONCLUSION

Many studies have reported ultrasonographic findings of fetuses affected with trisomy 18; however, the purpose of most studies was to diagnose trisomy 18 in the first or mid-trimester (Shields *et al.*, 1998; Jae *et al.*, 2005; Reinsch, 1997; Ronald *et al.*, 2003). To our knowledge, no study has examined the association between ultrasonographic findings in the prenatal period and neonatal/infant survival possibilities.

We investigated prenatal ultrasonographic findings of pregnant women who delivered live-born infants affected with trisomy 18. Our results showed that survival time less than 1 month was associated with severe polyhydramnios, absence of fluid in the stomach, severe cardiac anomaly and male sex. These findings could be useful for genetic counseling and decision making during pregnancies affected by this condition. The effect of intensive treatment including mechanical ventilation and surgery for cardiac/gastrointestinal malformation is yet to be determined.

## REFERENCES

Goldstein H, Nielsen KG. 1988. Rates and survival of individuals with trisomy 13 and 18. Data from a 10-year period in Denmark. *Clin Genet* **34**: 366–372.

Jae HY, Jin HC, Joong SS, June SC, Hyun MR, Moon YK. 2005. Prenatal diagnosis of trisomy 18: report of 30 cases. *Prenat Diagn* **25**: 119–122.

Kelly M, Robinson BW, Moore JW. 2002. Trisomy 18 in a 20-year-old woman. *Am J Med Genet A* **112**: 397–399.

Kosho T, Nakamura T, Kawame H, Baba A, Tamura M, Fukushima Y. 2006. Neonatal management of trisomy 18: clinical details of 24 patients receiving intensive treatment. *Am J Med Genet A* **140A**: 937–944.

Niedrist D, Riegel M, Achermann J, Schinzel A. 2006. Survival with trisomy 18-date from switzerland. *Am J Med Genet A* **140A**: 952–959.

Nyberg DA, Souter VL. 2001. Sonographic markers of fetal trisomies: second trimester. *J Ultrasound Med* **20**: 655–674.

Nyberg DA, Kramer D, Resta RG, *et al.* 1993. Prenatal sonographic findings of trisomy 18: review of 47 cases. *J Ultrasound Med* **2**: 103–113.

Reinsch RC. 1997. Choroid plexus cysts–association with trisomy: prospective review of 16,059 patients. *Am J Obstet Gynecol* **176**: 1381–1383.

Ronald W, Elizabeth T, Joe Leigh S, Eugene P, Richard S. 2003. First-trimester screening for trisomies 21 and 18. *N Engl J Med* **349**(15): 1405–1413.

Root S, Carey JC. 1994. Survival in trisomy 18. *Am J Med Genet* **49**: 170–174.

Shields LE, Carpenter LA, Smith KM, Nghiem HV. 1998. Ultrasonographic diagnosis of trisomy 18: Is it practical in the early second trimester? *J Ultrasound Med* **17**: 327–331.

Slavotinek A, Poyser L, Wallace A, Martin F, Gaunt L, Kingston H. 2003. Two unique patients with trisomy 18 mosaicism and molecular marker studies. *Am J Med Genet A* **117**: 282–288.

Sonja AR, Lee-Yang CW, Quanhe Y, Kristin MM, Friedman JM. 2003. Population-based analyses of mortality in trisomy 13 and trisomy 18. *Pediatrics* **111**: 777–784.

Taylor AI. 1968. Autosomal trisomy syndromes: a detailed study of 27 cases of Edwards' syndrome and 27 cases of Patau's syndrome. *J Med Genet* **5**: 227–252.

Tongsong T, Sirichotivakul S, Wanapirak C, Chanprapaph P. 2002. Sonographic features of trisomy 18 at midpregnancy. *J Obstet Gynaecol Res* **5**: 245–250.

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# Genital human papilloma virus infection in mentally-institutionalized virgins

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**Abstract**

*Objective.* Human papilloma virus (HPV) can cause cervical cancer. Risk factors for HPV infection are primarily related to sexual behavior. We determined the prevalence of HPV infection and abnormal cervical cytology in institutionalized women with no previous sexual experience.

*Methods.* The study subjects were 251 patients who sought screening for cervical cancer (45.9±9.4 years, mean±S.D., range, 14 to 66). They were institutionalized for psychosomatic disorders since childhood, and had no previous sexual experience. In addition to screening for cervical cancer, specimens for HPV testing were collected.

*Results.* No women who were positive for HPV DNA was detected, though 251 women without sexual experiences were screened by the hybrid capture 2 test including 26 types of HPV-DNA.

*Conclusion.* Transmission through means other than sexual intercourse may not exist because we could not detect HPV DNA in 251 women with no previous sexual experience.


*Keywords:* HPV; Virgins; Cervical cancer screening test; Prevalence of infection

## Introduction

The risk factors for HPV infection and cervical cancer are primarily related to sexual behavior, including number of sex partners, life time history of sex partners, and sexual behavior of prior sexual partners [1–4,6–8]. The current guidelines in the U.S. recommend women with normal recent cervical cytology without HPV to be screened every three years. However, how we should manage women with no previous sexual experience, e.g., virgins and physically handicapped women remains unknown, because previous studies have reported that HPV was virtually absent in women with no previous sexual experience [1,3]. So we designed the present study to determine the prevalence of HPV infection and cervical cancer in women with no previous sexual experience. We also discuss the necessity for determining the appropriate duration and frequency of follow-up screening of women with no previous sexual experience.

## Materials and methods

The study period spanned from September to December 2006, during which 251 patients who sought screening for cervical cancer were recruited. They had lived in an institution for individuals with psychosomatic disorders (Misakaeno-sono) since childhood, and had no previous sexual experience. They had undergone screening for cervical cancer annually or every 2 years. The results of screening for cervical cancer were normal for the past 12 years.

In this study, 89 conventional cervical cytology and 162 liquid-based cervical cytology (SurePath & CytoRoch, MBL) specimens were interpreted by cytopathologists. Interpretations were rendered by each cytopathologist according to his/her individual application of the diagnostic Bethesda System.

When the subjects underwent screening for cervical cancer, additional specimens for HPV test were collected by using Cytopick (Matsunami Glass Industries, Tokyo). Hybrid Capture 2 kit (Digene Corporation, Tokyo, Japan), which can detect HPV type 6, 11, 16, 18, 30, 31, 33, 34, 35, 39, 42, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 70 and 82, was used for HPV genotyping.

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All study protocols were approved by the Committee for Ethical Issues on Human Genome and Analysis of Nagasaki University.

## Results

During the study period, we evaluated 251 females ranging in age from 14 to 66 years (45.9±9.4 years, mean±S.D.). Their admission age were from 2 to 41 years old (15.9±7.8 years old, mean±S.D.). Their institutionalized period were from 2 to 41 years (30.3±8.8 years, mean±S.D.) before the current study. Their types of psychosomatic disorders are shown in Table 1. Virginity was confirmed in all participants by the presence of intact hymen and questionnaire or asking family members.

The results of cervical cytology screening test and HPV DNA test are shown in Table 2. Although 251 women without sexual experiences were screened by the hybrid capture 2 test including 26 types of HPV-DNA, no case of HPV-DNA positive was detected.

One woman was found to have abnormal cervical cytology (Table 2). Since swelling nucleus of parabasal cells were detected but the views suggesting HPV infection were not detected, her result of cytological screening was diagnosed as ASC-US by Bethesda System. She was negative for HPV DNA. She was 56 years old and completely asymptomatic. She was institutionalized for 37 years before the current finding.

## Discussion

In the present study, we showed that the prevalence of abnormal cervical cytology was 0.40% (1/251) among females with no previous sexual experience. The result of abnormal cytology detected was ASC-US by Bethesda System.

The low prevalence of abnormal cytology in our cohort with no previous sexual experience compared with females among the general population is probably related to the 12-year negative screening results for cervical cancer. However, we emphasize the necessity for regular follow-up because abnormal cytology was detected in one woman with no previous sexual experience.

Regarding the prevalence of HPV infection, no case of HPV positive was detected among Japanese women with no previous sexual experience surprisingly. Stevens-Simon et al. indicated that HPV is virtually absent in non-abused girls [2]. Furthermore, Gutman et al. suggested that HPV is absent in women with no previous sexual experience [1]. However non-sexual modes of transmission cannot be excluded, such as infection from humid dwellings, contaminated instruments and underwear, and vertical transmission from an infected mother to newborn babies [5].

Table 1
Main disease of the patients

| Disease | $n$=251 (%) |
|---|---|
| Mental retardation | 162 (64.5) |
| Cerebral palsy | 45 (17.9) |
| Sequela of the encephalitis or meningitis | 10 (4.0) |
| Chromosomal disorder | 15 (6.0) |
| Other diseases | 19 (7.6) |

Table 2
Results of cervical cytology screening test and HPV DNA test

| Bethesda system | $n$=251 (%) | Number of HPV-positive cases (%) |
|---|---|---|
| Negative | 250 (99.6) | 0 |
| ASC-US | 1 (0.4) | 0 |
| ASC-H | 0 (0) | 0 |
| LSIL | 0 (0) | 0 |
| HSIL | 0 (0) | 0 |
| SCC | 0 (0) | 0 |

Our results suggested that transmission through means other than sexual intercourse may not exist because we could not detect HPV DNA in patients with no previous sexual experience.

For the screening system of cervical cancer in women with no previous sexual experience how we should manage these population remains unknown. To answer this question, we need further follow-up studies to confirm whether the abnormal cervical cytology (the result of screening was ASC-US by Bethesda System) disappears or not in one woman. Up to the present, we did not detect abnormal cytology over 12 years in women with no sexual experience, and we could not detect HPV DNA in patients with no previous sexual experience. The data from this study supports that women with sexual experiences need to be screened longer because of the possibility of chronic infection with high-risk types of HPV. Meanwhile women without sexual experience probably do not need to be screened as often or as long, given the extreme unlikelihood of HPV infection in this patient population.

## References

[1] Gutman LT, St Claire K, Herman-Giddens ME, Johnston WW, Phelps WC. Evaluation of sexually abused and non-abused young girls for intravaginal human papillomavirus infection. Am J Dis Child 1992;146:694–9.
[2] Stevens-Simon C, Nelligan D, Breese P, Jenny C, Douglas Jr JM. The prevalence of genital human papillomavirus infections in abused and nonabused preadolescent girls. Pediatrics 2000;106:645–9.
[3] Kari S, Stima S. Infections in Human Pathology; 1995. p. 117–26.
Critchlow CW, Koutsky LA. Epidemiology of human papillomavirus infection. In: Mindel A, editor. Genital Warts. Human papillomavirus Infection. Kent: Edward Arnold; 1995. p. 53–81.
[4] Schlecht NF, Kulaga S, Robitaille J, et al. Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia. JAMA 2001;286: 3106–14.
[5] Cason J, et al. Perinatal infection and persistence of human papillomavirus types 16 and 18 in infants. J Med Virol 1995;47:209–18.
[6] Jacobs MV, Snijders PJ, van den Brule AJ, Helmerhorst TJ, Meijer CJ, Walboomers JM. A general primer GP5+/GP6(+)-mediated PCR enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. J Clin Microbiol 1997;35:791–5.
[7] Winer RL, Lee SK, Hughes JP, Adam DE, Kiviat NB, Koutsky LA. Genital human papillomavirus: infection incidence and risk factors in a cohort of female university students. Am J Epidemiol 2003;157:218–26.
[8] Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998;338:423–8.

## Circulating Cell-Free Placental mRNA in the Maternal Plasma as a Predictive Marker for Twin-Twin Transfusion Syndrome

*To the Editor:*

Twin-twin transfusion syndrome (TTTS), which is a serious complication in monochorionic diamniotic twins (MCDA-T), involves unequal blood flow via the placental vascular anastomoses from the donor to the recipient twin. Although the placental anastomoses are present in all MCDA-T and both fetuses are genetically identical, TTTS occurs in only 15% of MCDA-T, and much of the pathophysiological basis of TTTS remains poorly understood. Clinically, a staging system based on the ultrasound features of TTTS is widely used for the management *(1)* but not for the prediction of TTTS. In addition, the known predictive findings observable by ultrasonographic examination are detectable only in a small portion of TTTS cases *(2)*. New predictive markers are therefore desirable for the early detection and prevention of TTTS. Recently, placental mRNAs, such as human placental lactogen (PL) and some other hormones were detected in maternal plasma, and concentrations of each marker were measured with quantitative real-time reverse transcription (RT)-PCR *(3, 4)*. Thus, circulating cell-free mRNA (cf-mRNA) in maternal plasma has become an attractive target for the noninvasive monitoring of pregnancy disorders *(3, 5)*.

The purpose of the present study was to investigate the use of cf-mRNA concentration in maternal plasma as a predictive marker of later TTTS. The study participants included 17 pregnant women who visited the Obstetrics Clinic of Nagasaki University Hospital at 12–21 weeks of gestation for management of their pregnancy with MCDA-T. Included as a control group were 135 singleton pregnant women without medical complications at similar gestational age. All of the participants gave written informed consent, and the study was approved by the Research Ethics Committee of Nagasaki University. Although none of the 17 cases of MCDA-T were complicated by TTTS at the time of blood sampling, TTTS subsequently developed in 5 cases (TTTS group), but not in the remaining 12 cases (no-TTTS group). Gestational ages at diagnosis of TTTS were 15–25 weeks. The 3 groups had no significant differences in population characteristics, including the maternal age, the number of nulliparous women, and the gestational age at the time of sampling (data not shown).

The blood samples (8 mL) from each woman were collected into an EDTA tube, and the plasma sample was stored at −20 °C until use. After cf-mRNA was extracted from maternal plasma, a quantitative 1-step real-time RT-PCR assay was performed using an ABI 7900T Sequence Detector (Perkin-Elmer) as described previously *(4)*. Primer sets and TaqMan probes for each gene and single-strand, and synthetic DNA oligonucleotides from each amplicon used for a calibration curve were prepared as described previously *(4)*. Then, plasma concentrations of cf-mRNA for human PL and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured and converted into multiples of the median (MoM) of the controls adjusted for gestational age, as described previously *(5)*. The differences between the TTTS and the no-TTTS groups were evaluated with the Mann–Whitney *U*-test. Significant difference was defined as a $P$ value $<0.05$.

The median (minimum–maximum) cf-PL mRNA MoM values were 1.80 (0.89–3.81) in the TTTS-group, 1.14 (0.77–1.35) in the no-TTTS group, and 1.00 (0.82–2.05) in the control group, respectively. At adjusted gestational age the cf-*PL* mRNA concentration was significantly higher in the TTTS group than in the no-TTTS group (Mann–Whitney *U*-test, $P = 0.035$), whereas there was no significant difference of cf-*PL* mRNA concentration between the no-TTTS group and the control group ($P = 0.41$; Fig. 1). In addition, the median cf-GAPDH mRNA MoM value in the maternal plasma was significantly higher in the TTTS

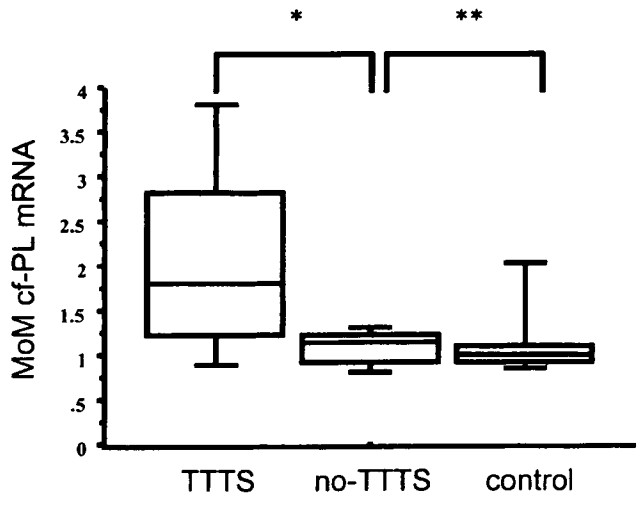


Fig. 1. Box and whiskers plots of cf-PL MoM distribution in the TTTS group, no-TTTS group, and control group.

The median (minimum–maximum) cf-PL mRNA MoM values were 1.80 (0.89–3.81) in the TTTS group, 1.14 (0.77–1.35) in the no-TTTS group, and 1.00 (0.82–2.05) in the control group. * $P$ = 0.035, ** $P$ = 0.41.

group (2.20; range 1.30–2.68) than in the no-TTTS group (1.09; range 0.68–3.25; $P$ = 0.045). Our results suggested the possibility that unapparent pathophysiological changes had already occurred in the women who subsequently developed TTTS, although which specific conditions led to the increased mRNA in the maternal plasma in the TTTS group remain unknown.

In conclusion, a quantitative aberration of both the cf-PL and cf-GAPDH mRNA in maternal circulation may be a novel predictive marker for TTTS, although both statistical differences were small and the sample size was too small to give sufficient strength to the analysis. Therefore, a combination of several cell-free placental mRNA markers could be effective for the prediction of TTTS, similar to the situation for tumor markers. Further study to identify gene transcripts that are expressed only in the placenta and not in blood cells may help to both predict and prevent TTTS and also may further elucidate the pathophysiology of this serious complication.

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**References**

1. Quintero RA, Morales WJ, Allen MH, Bornick PW, Johnson PK, Kruger M. Staging of twin-twin transfusion syndrome. J Perinatol 1999;19:550–5.
2. Jain V, Fisk NM. The twin-twin transfusion syndrome. Clin Obstet Gynecol 2004;47:181–202.
3. Dennis Lo YM, Chiu RW. Prenatal diagnosis: progress through plasma nucleic acids. Nat Rev Genet 2007;8:71–7.
4. Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS, et al. mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 2003;100:4748–53.
5. Purwosunu Y, Sekizawa A, Koide K, Farina A, Wibowo N, Wiknjosastro GH, et al. Cell-free mRNA concentrations of plasminogen activator inhibitor-1 and tissue-type plasminogen activator are increased in the plasma of pregnant women with preeclampsia. Clin Chem 2007;53:399–404.

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