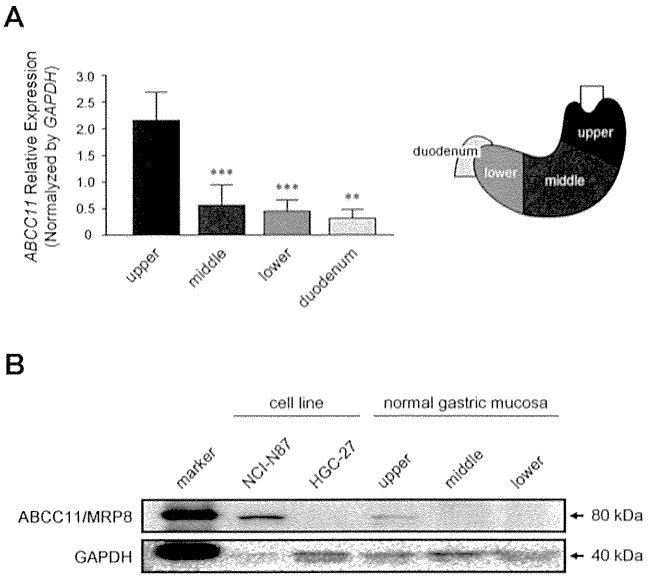

Fig. 5. Localization of ABCC11/MRP8 in the normal gastric mucosa was investigated using specimens from surgically resected patients. **A:** Quantitative real-time PCR analysis of 19 specimens for four parts of the normal gastric mucosa: upper, cardia, and fundus; middle, body of stomach; lower, pyloric antrum and pyloric canal; and duodenum, superior part of the duodenum. The right schematic shows the definitions of each part of the stomach. Data represent the mean±SD. Statistical significance was determined by unpaired Student's *t*-test. **, $P<0.01$ and ***, $P<0.001$. **B:** Western blot analysis of gastric cancer cell lines (NCI-N87 and HGC-27) and normal gastric specimens from four parts of the gastric mucosa. GAPDH was used as the internal control protein.

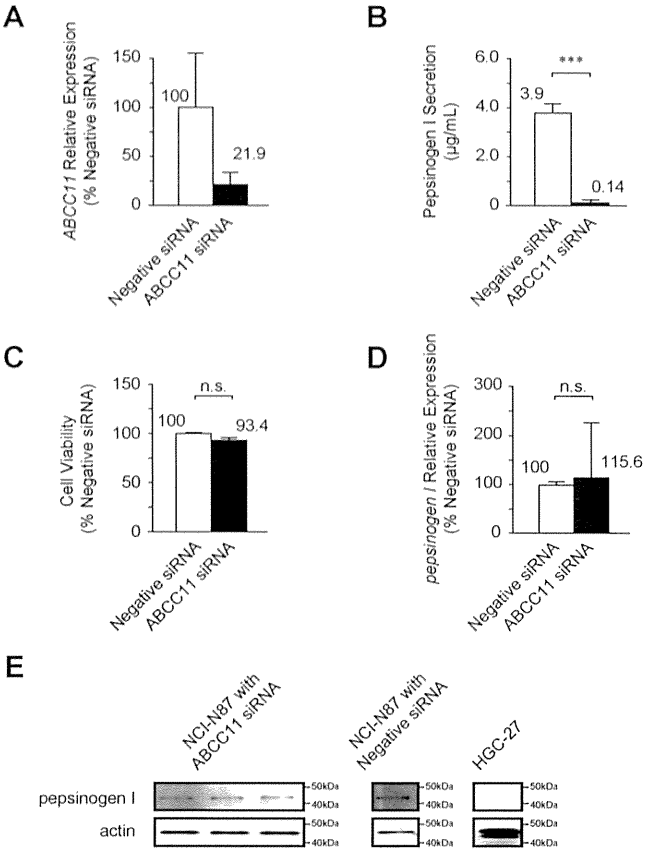

Fig. 6. Alteration of pepsinogen I secretion by ABCC11 siRNA was investigated using NCI-N87 cells. **A:** Quantitative real-time PCR analysis of *ABCC11* mRNA two days after siRNA transfection. Data were normalized to housekeeping gene *GAPDH* mRNA. Graph shows the relative *ABCC11* expression compared with negative siRNA as 100%. Data represent mean±SD. **B:** Secreted pepsinogen I in each culture medium measured by ELISA two days after siRNA transfection. Graph shows relative pepsinogen I secretion compared with negative siRNA as 100%. Statistical significance was determined by unpaired Student's *t*-test. ***, $P<0.001$. **C:** Cell viability was evaluated by WST-8 assay to investigate the cytotoxicity associated with the transfection agents or manipulations at two days after transfection. Graph shows the relative viability compared with negative siRNA as 100%. Data represent the mean±SD. Statistical significance was determined by unpaired Student's *t*-test. n.s., not significant. **D:** Quantitative real-time PCR analysis of *pepsinogen 1* mRNA at two days after siRNA transfection. Data were normalized to housekeeping gene *GAPDH*. Graph shows the relative *pepsinogen 1* mRNA expression compared with negative siRNA as 100%. Data represent the mean±SD. **E:** Western blot analysis for pepsinogen I. Cytoplasmic proteins from each fraction of NCI-N87 cells transfected with ABCC11 siRNA were extracted at two days after transfection in triplicate. NCI-N87 cells transfected with negative siRNA were used as a positive control, and HGC-27 cells were used as a negative control. Actin was used as the internal control protein.

lators that bind the gastric chief cell receptors with specific antagonists have been studied intensively. However, the mechanism by which they are sequestered into granules and moved to the plasma membrane for exocytosis has not been identified. In the present study, *ABCC11*-knocked down cells could synthesize pepsinogen I intracellularly, but failed to secrete it extracellularly. Considering the molecular weight of the pepsinogen I, it may be too large for a transportation substrate of ABCC11/MRP8 [36]. Thus, the mechanism of reducing pepsinogen I with ABCC11-knockdown might be an indirect suppression, through some smaller molecule essential to the generating process of pepsinogen I. Although further research is necessary for elucidation of the mechanisms, our results suggest that ABCC11/MRP8 might play a crucial role in the preliminary steps of pepsinogen I exocytosis.

Previously, we reported the functional modification of ABCC11/MRP8 that determined the phenotype of earwax by a SNP of the ABCC11 gene [35]. Similarly, ABCC11 genotype is responsible for the secretion in the axillary apocrine gland [29], and axillary osmidrosis [11, 20, 30]. In addition, ABCC11 genotype is also responsible for secretion of colostrum [20]. Considering our experimental data and the studies cited here, ABCC11/MRP8 might be a key molecule of "exocrine" function in various tissues. It is still unknown whether the SNP genotype correlates with pepsinogen I secretion. Serum pepsinogen tests were introduced for mass screening of chronic gastritis and gastric cancer [15, 17–19]. Therefore, it would be clinically important to investigate the SNP genotype and pepsinogen I. Additional studies of ABCC11/MRP8 including SNP genotyping and secretion models might help to clarify the mechanism of exocrine systems in the gastrointestinal tract.

In conclusion, low expression of ABCC11/MRP8 was

found in gastrointestinal tract cancers. Our findings would have some implications for the rational understanding of chemoresistance in gastrointestinal tract cancers. Furthermore, our findings also indicate a novel function of ABCC11/MRP8 in normal gastric chief cells, which regulate pepsinogen I secretion, possibly in the process of exocytosis. These results might give us a new insights into pepsinogen production and ABCC11/MRP8 regulation in gastric diseases.

V. Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

VI. Acknowledgments

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De novo SOX11 mutations cause Coffin–Siris syndrome

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Coffin–Siris syndrome (CSS) is a congenital disorder characterized by growth deficiency, intellectual disability, microcephaly, characteristic facial features and hypoplastic nails of the fifth fingers and/or toes. We previously identified mutations in five genes encoding subunits of the BAF complex, in 55% of CSS patients. Here we perform whole-exome sequencing in additional CSS patients, identifying *de novo SOX11* mutations in two patients with a mild CSS phenotype. *sox11a/b* knockdown in zebrafish causes brain abnormalities, potentially explaining the brain phenotype of CSS. SOX11 is the downstream transcriptional factor of the PAX6–BAF complex, highlighting the importance of the BAF complex and SOX11 transcriptional network in brain development.

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Coffin–Siris syndrome (CSS; MIM#135900) is a congenital disorder characterized by growth deficiency, intellectual disability, microcephaly, characteristic facial features and hypoplastic nails of the fifth fingers and/or toes (Supplementary Fig. 1). Five subunit genes (*SMARCB1*, *SMARCA4*, *SMARCE1*, *ARID1A* and *ARID1B*) of the BAF complex (also known in yeast as the SWI/SNF complex[1]) are mutated in 55–70% of CSS patients[2–6]. Mutations in *SMARCA2*, another BAF complex gene, were reported in the Nicolaides–Baraitser syndrome, which is similar to, but distinct from CSS[7]. Furthermore, *de novo PHF6* mutations were found in two CSS patients[6], although no direct interaction has been reported between the BAF complex and PHF6, which interacts with the nucleosome remodelling and deacetylation complex[6]. As 30–45% of CSS patients were genetically undiagnosed in three large cohort studies[2–6], further genetic investigation is required to fully address the genetic picture of CSS.

Here we apply whole-exome sequencing (WES) to 92 CSS patients, and identify two *de novo SOX11* mutations in two unrelated patients. *sox11* knockdown experiments in zebrafish result in a smaller head and significant mortality, which were partially rescued by human wild-type *SOX11* messenger RNA (mRNA), but not by mutant mRNA.

Results

WES of CSS patients. We identified two *de novo SOX11* mutations in two unrelated patients, c.347A>G (p.Tyr116Cys) (in patient 1) and c.178T>C (p.Ser60Pro) (in patient 2) (deposited to LOVD, http://www.LOVD.nl/SOX11), among 92 CSS patients (including our previous cohorts[2,3]) analysed by trio-based WES. In the two patients, >10 reads covered 94–92% of coding sequences and only *SOX11* mutations remained as candidate variants in both of them based on the *de novo* model with scores of damaging or disease causing by SIFT, PolyPhen2 and Mutation Taster (Supplementary Table 1). The two heterozygous mutations localize to the high-mobility group (HMG) domain. Neither mutation was registered in the databases examined (1,000 Genomes, Exome Sequencing project (ESP) 6500, and in-house databases containing 575 control exomes) (Supplementary Table 1). We identified a further 22 *SOX11* variants within these three databases, but all of them reside outside the HMG domain and, based on prediction programs, are less likely to be pathogenic (Supplementary Table 1; Supplementary Fig. 2). The amino acids altered in SOX11 are evolutionarily conserved from zebrafish to human (Fig. 1). The mutations do not alter nuclear localization of SOX11 protein (Supplementary Fig. 3). *De novo* mutations were confirmed in the two families by Sanger sequencing along with biological parentage. No mutations in any of the other BAF complex genes, *PHF6*, or other potential candidate genes were found in the two families. Therefore, the two mutations identified are highly likely to be pathogenic. Moreover, *SOX11* was sequenced by WES ($n=23$) or Sanger method ($n=67$) in a further 90 CSS patients, with no mutations found. Fifty-four patients had a mutation in one of the five BAF complex subunit genes (58.7%) (*SMARCA4*, *SMARCB1*, *SMARCE1*, *ARID1A* and *ARID1B* mutations found in 9, 8, 1, 5 and 31 patients, respectively).

Figure 1 | *SOX11* mutations and functional characterization. *SOX11* mutations in CSS patients. Two missense mutations in the HMG domain (blue box) occur at evolutionarily conserved amino acids.

Clinical features of patients with *SOX11* mutations. The two patients showed dysmorphic facial features, microcephaly, growth deficiency, hypoplastic fifth toe nails and mild intellectual disability[8] (Supplementary Fig. 1; Supplementary Table 2). The observed clinical features in both patients are classified to a mild end of CSS as patient 1 spoke early for CSS and patient 2 has relatively high intelligence quotient. Although the two patients do not look similar in facial appearance (patient 1 has midface hypoplasia, while patient 2 does not; in addition there is an ethnic difference, as patients were either Japanese or Indian), they do share features in common, namely, hypertrichosis, arched eyebrows, low-set ears, auricular back-rotationand full cheeks (Supplementary Fig. 1).

Patient 1 (Japanese) was born at 38 weeks of gestation following an uneventful pregnancy. Her birth weight was 2,340 *g* (−1.9 s.d.), length 45 cm (−2.2 s.d.) and occipitofrontal circumference (OFC) 30.5 cm (−1.8 s.d.). She was hypotonic, had feeding difficulties (especially during the neonatal period) and delayed development. She was able to support her head at 5 months of age, sit at 11 months and walk independently at 1 year 11 months. She started to speak meaningful words at 1 year 7 months. At 3 years, her developmental quotient was estimated using the Kyoto scale of psychological development to be 57. Abdominal echography showed her left kidney was slightly small in size. She has distinctive facial features characterized by midface hypoplasia, short palpebral fissures, hypertelorism, upturned palpebral fissures, long eyelashes, a low nasal root, shortened nose with upturned nostrils, short philtrum, open mouth, full lips and low-set ears. Hypoplastic distal phalanges with nail hypoplasia (especially of the fifth digits) were also noted. Additional findings included hypertrichosis and long eyelashes with abundant hair on the scalp. At 4 years 8 months, she was short with a height of 92.1 cm (−2.9 s.d.) and evaluated for possible growth hormone deficiency with stimulation tests, which showed normal results. At 10 years, she measured 119 cm (−2.8 s.d.), weighed 20.1 kg (−1.8 s.d.) and had an OFC of 47.3 cm (−3.3 s.d.). She attends a special education class for poor performance, but can walk to school by herself (takes approximately half an hour) and is able to communicate verbally, to some extent, with her classmates. Clinical features are summarized in Supplementary Table 2.

Patient 2 (Indian) is a 16-year-old female, and was referred to the genetics outpatient department for evaluation of short stature. She was born at term following a normal pregnancy, but with low birth weight (1.75 kg, −4s.d.). Developmental milestones were attained normally, but her parents always felt that she lagged behind other children. She was a slow learner with poor scholastic performance and an intelligence quotient of 70–80. She attended a normal class, but struggled to pass class examinations every year. She has a proportionately short stature but not a coarse face. Her chin was small and supraorbital ridges hypoplastic with no ptosis. Her nose was long and alae nasi hypoplastic with overhanging columella. Her hair was thick and rough with some thinning on her scalp. She had increased hair on her back. Her fourth and fifth toes were short and all her finger nails were hypoplastic with thin and tapered fingers. Her fourth and fifth toes on both feet, and also the third toe on her right foot, were

markedly hypoplastic. Clinodactyly was noted on the third, fourth and fifth toes on her right foot, and the third and fourth toes on her left foot. A skeletal survey did not show any radiographic bone abnormalities. Her bone age was 13–14 years and follicle-stimulating hormone was 1.57 IU l^{-1} (normal range: <5 IU l^{-1}). Ultrasonographic examination at 16 years (before menarche), showed a hypoplastic uterus and malrotation of both kidneys. No secondary sexual characteristics were recognized until she had menarche at 17 years. Now at age of 17 years, she is still short with a height of 141 cm (−5 s.d.), weigh 31.3 kg (−3 s.d.) and OFC 50.5 cm (−4.5 s.d.). Clinical features are summarized in Supplementary Table 2.

Structural effects of SOX11 mutations. To determine the impact of the disease-causing mutations on human SOX11 structure and function, we mapped the mutation positions onto the crystal structure of mouse Sox4[9], that is analogous to human SOX11, and calculated free energy changes on the mutations using FoldX software[10,11]. The mutations lie in the highly conserved HMG domain, responsible for sequence-specific DNA binding (Fig. 2a)[9]. Ser60 is located in a helix of the HMG domain (Fig. 2a), therefore the S60P mutation may affect overall folding of the HMG domain and impair DNA binding of SOX11. FoldX calculations supported this prediction and the free energy change on the mutation was high enough to destabilize protein folding (>10 kcal mol^{-1}) (Fig. 2b)[12]. Conversely, Tyr116 forms a hydrophobic core with the side chains of DNA-recognition loops (Fig. 2a). The Y116C mutation has low free energy change (<1 kcal mol^{-1}) (Fig. 2b), and is unlikely to significantly affect folding of the HMG domain, but instead may alter conformation of the DNA-recognition loop, which is important for DNA binding.

***SOX11* mutations affect downstream transcription**. Both mutations are located within the HMG domain, which is required for *SOX11* transcriptional regulation of *GDF5* (ref. 13). Luciferase assays using the *GDF5* promoter in HeLa and ATDC5 cells, showed both mutant proteins had decreased transcriptional activities compared with wild type (WT) (Fig. 3).

SOX11 expression. *SOX11* transcription levels were examined using multiple human complementary DNA (cDNA) panels. *SOX11* was exclusively expressed in brain (foetus and adult) and heart (adult) tissues, supporting a role for *SOX11* mutations in the brain features of CSS observed in the two patients (Supplementary Fig. 4; Supplementary Table 2).

In mice, targeted *Sox11* disruption with a β-galactosidase marker gene results in 23% birth weight reduction and lethality after the first postnatal week in homozygotes, due to hypoplastic lungs and ventricular septation defects. In addition, skeletal malformations (including phalanges) and abdominal defects are observed[14]. Physical and functional abnormalities in heterozygotes have not been described. However, in heterozygous mice, β-galactosidase expression revealed early ubiquitous expression throughout the embryo with upregulation in the central nervous system (CNS) and limb buds[14].

***sox11* knockdown experiments in zebrafish**. We further investigated *sox11* function in zebrafish. The zebrafish genome contains two orthologs of human *SOX11*, *sox11a* and *sox11b*, which are expressed in all cells until gastrulation and later become restricted to the developing CNS[15,16]. We knocked down zebrafish *sox11a* and *sox11b* (both single-exon genes) using translation-blocking morpholino oligonucleotides (MOs) (*sox11a*-MO, *sox11b*-MO and *sox11a/b*-MO), as previously described[17] (Supplementary Fig. 5a). Off-target effects of morpholino injections were excluded by repeated experiments, co-injecting with *tp53* MO or injecting into *tp53*$^{zdf1/zdf1}$ mutant fish[18,19]. *sox11a*- and *sox11b*-MO knockdown caused similar phenotypes, including smaller heads and body curvature (Supplementary Fig. 5b). Low-dose *sox11a*- (1.6 ng), *sox11b*- (1.6 ng) and *sox11a/b*- (1.6 ng) MO-injected embryos resulted in

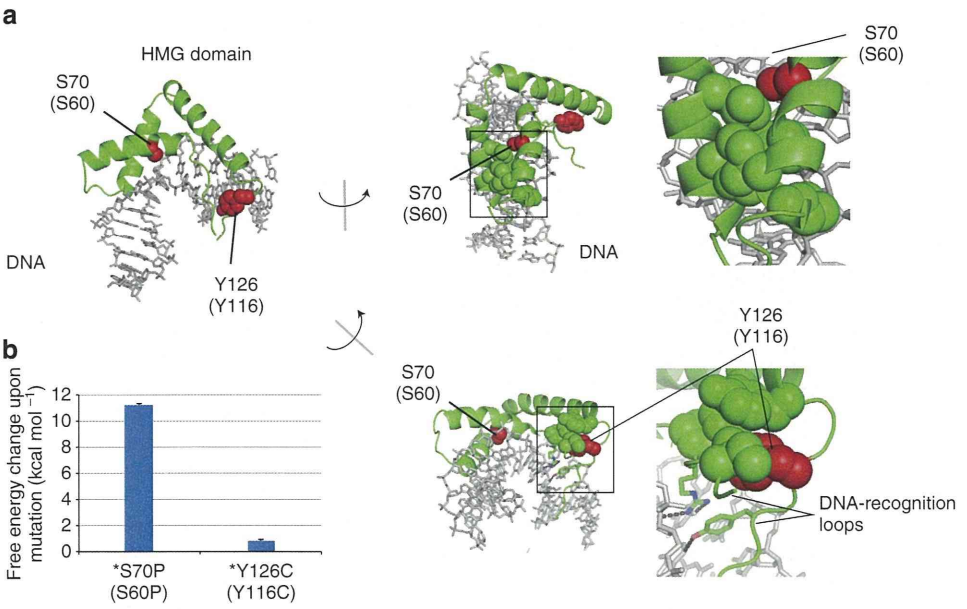

Figure 2 | Structural effects of SOX11 mutations. (**a**) Crystal structure of the mouse Sox4 HMG domain bound to DNA. Helices and loops are shown as green ribbons and threads, respectively. DNA is shown as grey sticks. Amino-acid residues at mutation sites are shown coloured red in the space-filling model. In the middle and right images, some of the amino-acid residues involved in the hydrophobic core surrounding mutation points are shown coloured green in the space-filling model. Amino-acid numbering is indicated for mouse Sox4 with that for human SOX11 in parentheses. Hydrogen bonds are shown as black dotted lines. Molecular structures were drawn using PyMOL (http://www.pymol.org). (**b**) Free energy changes on the indicated mutations calculated by FoldX software.

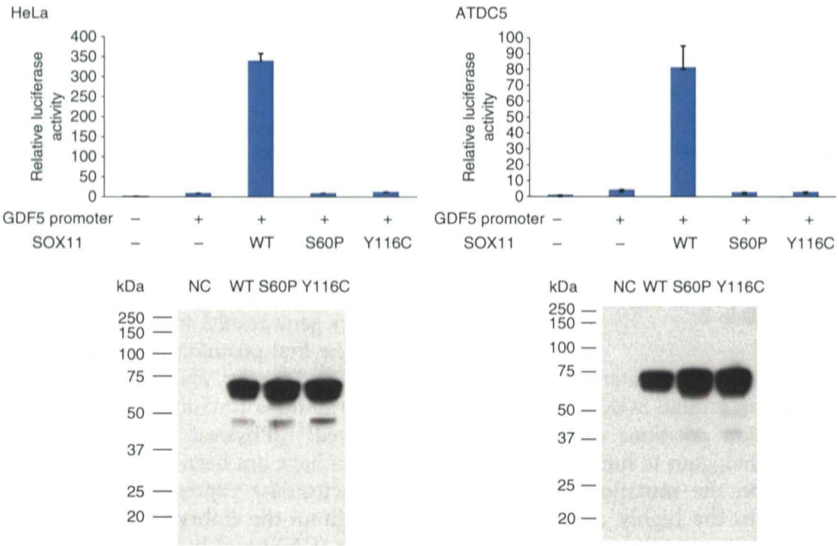

Figure 3 | *SOX11* mutations affecting *GDF5* promoter activity. Luciferase reporter assays measured transcriptional activity of the *GDF5* promoter (−448/+319) (UCSC genome browser hg19: chr20: 34025709-34026457) in HeLa (left) and ATDC5 (right) cells. HeLa or ATDC5 cells were co-transfected with WT or mutant (S60P and Y116C) SOX11 expression vector and reporter constructs containing either GDF5 promoter or empty vector (pGL3-basic). Relative luciferase activities compared with empty vector are presented as mean ± s.d. for two independent experiments, with each experiment performed in triplicate (upper). Immunoblot analysis of transfected HeLa and ATDC5 cell extracts showing wild-type (WT) or mutant (S60P and Y116C) SOX11 proteins (lower). Compared with WT, both SOX11 mutants reduced *GDF5* transcriptional activities in HeLa and ATDC5 cells.

significant mortality (*sox11a*-MO, ~49.3%; *sox11b*-MO, ~19.3%; *sox11a/b*-MO, ~53.0%), compared with control-MO embryos (~7.3%) (Fig. 4a). Co-injection of WT human *SOX11* mRNA (h*SOX11*-WT mRNA) with *sox11a/b*-MO improved morphant survival at 48 h post fertilization (hpf) (25.5% lethality versus 49.3% lethality with *sox11a/b*-MO alone) ($P<0.01$) (Fig. 4a; Supplementary Fig. 5c). The affected phenotype of *sox11a/b* double morphants was partially rescued by h*SOX11*-WT mRNA overexpression (4.5% normal for *sox11a/b*-MO alone versus 36.5% for co-injection with h*SOX11*-WT mRNA and *sox11a/b*-MO, $P<0.01$) (Fig. 4a). In contrast, co-injection of either mutant h*SOX11* mRNA (h*SOX11*-S60P and -Y116C mRNA) with *sox11a/b*-MO showed no significant rescue effects on lethal and affected phenotypes (Fig. 4a). There were significantly more normal phenotypes following h*SOX11*-WT mRNA and *sox11a/b*-MO co-injection, than with h*SOX11*-mutant mRNA co-injections ($P<0.05$). Head sizes in randomly selected embryos ($n\geq10$) of *sox11a* and *sox11a/b* morphants at 48 hpf were significantly decreased ($P<0.05$ in both), but not significantly changed in *sox11b* morphant. Overexpression of h*SOX11*-WT mRNA restores *sox11a/b* double-morphant head size (in randomly selected embryos, $n\geq10$), suggesting specific *sox11* suppression by morpholino injection (Fig. 4b). Although the head size of h*SOX11*-mutant mRNA and *sox11a/b*-MO-injected embryos was slightly decreased, no significant difference was recognized between overexpression of h*SOX11*-WT or h*SOX11*-mutant mRNA and *sox11a/b*-MO co-injection (Fig. 4b).Staining with acridine orange and terminal deoxynucleotidyl TdT-mediated dUTP nick end labelling (TUNEL), found significant apoptotic increases exclusively in microcephalic embryos (Fig. 4c; Supplementary Fig. 6). Brain cell death was prevented by co-injection with h*SOX11*-WT mRNA, but not by mutant h*SOX11* mRNAs (Fig. 4c). We also used HuC/D (a marker for early postmitotic and mature neurons) and acetylated tubulin (an axonal marker) immunostaining at 48 hpf to analyse neuronal cells in more detail (Supplementary Fig. 7). Decreased HuC/D-positive neurons, especially in the telencephalon and diencephalon, were observed in *sox11* morphants (Supplementary Fig. 7a). The phenotype in *sox11a/b*-MO-injected embryos was efficiently rescued by h*SOX11*-WT mRNA (Supplementary Fig. 7a). Reduction of HuC/D-positive neurons was unaltered by mutant h*SOX11* mRNA overexpression and *sox11a/b*-MO injection (Supplementary Fig. 7a). Anti-acetylated tubulin staining also showed severely reduced axonal numbers in the forebrain, midbrain and hindbrain of *sox11* morphants, compared with control-MO-injected embryos (Supplementary Fig. 7b). *sox11a/b* morphants showed phenotypic rescue when co-injected with h*SOX11*-WT mRNA, compared with mutant h*SOX11* mRNAs (Supplementary Fig. 7b).

Discussion

We have identified *SOX11* mutations in CSS. This is the first report of human mutations in *SOXC* (*SOX4*, *SOX11* and *SOX12*)[20]. *SOX11/sox11* is required for neurogenesis, and loss of function in early embryos is sufficient to impair normal CNS development. Haploinsufficiency of other *SOX* genes (*SOX2*, *SOX9* and *SOX10*) is known to cause human diseases[21–23]. It is interesting that mutations of *SOX11* and other BAF subunit genes are mutually exclusive in CSS.

Sox11 was recently shown to form a transcriptional cross-regulatory network downstream of the Pax6–BAF complex. The network drives neurogenesis and converts postnatal glia into neurons[24]. Brg1 (Smarca4) binds to the *Sox11* promoter, and interaction with Pax6 is sufficient to induce Sox11 expression in neurosphere-derived cells in a Brg1-dependent manner[24]. Therefore, the Pax6–BAF complex activates a cross-regulatory transcriptional network, maintaining high expression of genes involved in neuronal differentiation and execution of cell lineage decisions[24]. *SOX11* mutations appear to be a rare cause of CSS as only 2 out of 92 patients (2.2%) showed *SOX11* abnormality and to be limited to the mild end of CSS phenotype. Abnormality of the upstream BAF complex tends to show a more severe phenotype compared with that of a downstream *SOX11* mutation, which may indicate rather specific effects of *SOX11* mutations on the CSS phenotype.

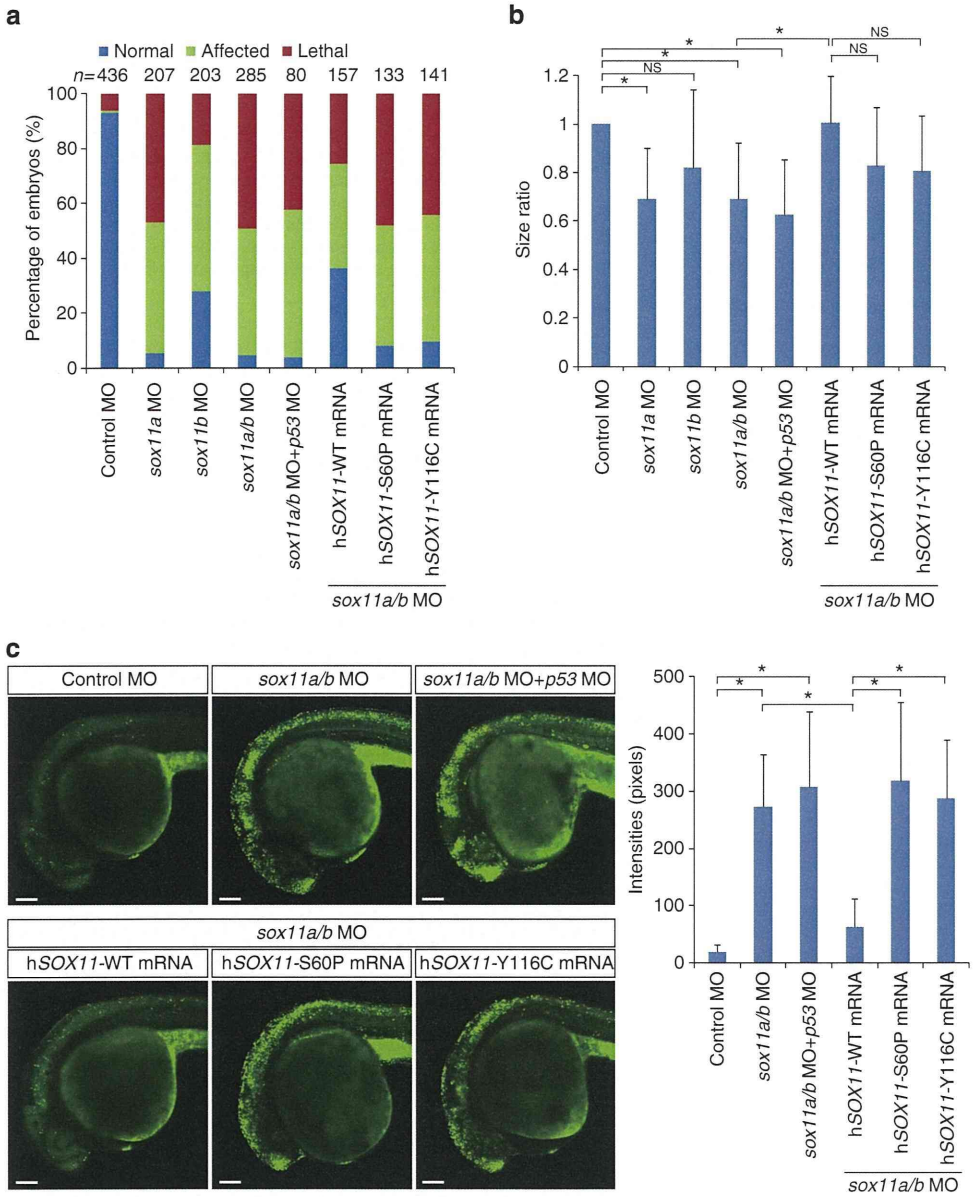

Figure 4 | *sox11a/b* knockdown experiments in zebrafish. (**a**) Embryos were injected with *sox11*-MO alone or with *sox11*- and *tp53*-MO or with *sox11*-MO and *in vitro* transcribed human *SOX11* (h*SOX11*) mRNA (WT, wild type; S60P, p.Ser60Pro; Y116C, p.Tyr116Cys). Injected embryos were categorized as normal, affected and lethal at 48 hpf. The lethal and affected phenotype in *sox11a/b*-MO-injected embryos was partially rescued by WT h*SOX11* mRNA overexpression. All experiments were performed more than twice and evaluated statistically with a Student's *t*-test. (**b**) Head size ratios of embryos with control-, *sox11a*-, *sox11b*- or *sox11a/b*-MO alone, or with *sox11a/b*- and *tp53*-MO or *sox11a/b*-MO and h*SOX11* mRNA (WT or mutant) at 48 hpf ($n \geq 10$) (average of control-MO as 1). Dorsal views of midbrain width were measured. Data are represented as mean ± s.d. $^*P < 0.05$ by Student's *t*-test. NS, not significant. (**c**) Brain cell death in MO-injected embryos at 30 hpf using acridine orange staining (lateral view). *sox11* morphants show increased cell death in the CNS. Scale bar, 100 μm. Quantification of acridine orange intensities in morphants are shown graphically (right, $n \geq 10$). Data are represented as mean ± s.d. $^*P < 0.001$ by Student's *t*-test.

In conclusion, mutations in both BAF complex genes and *SOX11* result in the same phenotype (CSS), providing strong support for the BAF complex and SOX11 function in a common pathway, and play an important role in human brain development.

Methods

Subjects and clinical data. Patients were seen by their attending clinical geneticists. DNA samples were isolated from peripheral blood leukocytes using standard methods. Informed consent was obtained from the parents of the patients for experimental protocols and displaying participants' facial appearances in publications. This study was approved by the institutional review board of Yokohama City University School of Medicine. A total of 92 patients were analysed, including 71 patients from a previous cohort and 21 new patients.

WES. Trio-based WES was performed in two families. Briefly, 3 μg of genomic DNA was sheared using the Covaris 2S system (Covaris, Woburn, MA) and partitioned using SureSelect Human All Exon V4 or V4 + UTRs (Agilent Technology, Santa Clara, CA), according to the manufacturer's instructions. Exon-enriched DNA libraries were sequenced using HiSeq2000 (Illumina, San Diego, CA) with 101-bp paired-end reads and 7-bp index reads. Four samples (2.5 pM each, with different indexes) were run in one lane. Image analysis and base calling were performed using HiSeq Control Software/Real-Time Analysis and CASAVA1.8.2 (Illumina). Mapping to human genome hg19 was performed using Novoalign (http://www.novocraft.com/main/page.php?s=novoalign). Aligned reads were

processed by Picard (http://picard.sourceforge.net) to remove PCR duplicates. Variants were called using the Genome Analysis Toolkit 1.5–21 (GATK v3) with best practice variant detection (http://gatkforums.broadinstitute.org/discussion/15/best-practice-variant-detection-with-the-gatk-v1-x-retired), and annotated by Annovar (23 February 2012) (http://www.openbioinformatics.org/annovar/). Common variants registered in dbSNP137 (MAF $\geq$ 0.01) (http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=281702941&c=chr1&g=snp137Flagged) were removed.

Prioritization of variants. From all the variants within exons and $\pm$2 bp of intronic regions from exon–intron boundaries, those registered in either dbSNP137, 1,000 Genomes (http://www.1000genomes.org/), ESP 6500 (http://evs.gs.washington.edu/EVS/) or our in-house (exome data from 408 individuals) databases, and those located within segmental duplications, were removed and we focused on heterozygous non-synonymous and splice site variants, which were subsequently confirmed by Sanger sequencing. *SOX11* mutations in LOVD, http://www.LOVD.nl/SOX11.

Structural modelling and free energy calculations. The crystal structure of the mouse Sox4 HMG domain bound to DNA (Protein Data Bank code 3U2B) was selected by SWISS-MODEL server 5 (ref. 25) as the structure most resembling human SOX11. To examine the missense mutations, mutational free energy changes were calculated using FoldX software (version 3.0)[10,11]. Calculations were repeated three times, and resultant data presented as average values with s.d.

***SOX11* expression analysis in human tissues.** TaqMan quantitative real-time PCR was performed using cDNAs from adult (Human MTC Panel I, #636742, Clontech Laboratories, Mountain View, CA) and foetus (Human Fetal MTC Panel, #636747, Clontech Laboratories). Pre-designed TaqMan probes for human *SOX11* (Hs00167060_m1, Life Technologies Co., Carlsbad, CA) and human beta-actin (ACTB, 4326315E, Life Technologies Co.) were used. PCR was performed on a Rotor-Gene Q (QIAGEN, Valencia, CA) and expression levels normalized to *ACTB*, an internal standard gene, according to the $2^{-\Delta\Delta Ct}$ method. Kidney expression was used as the standard ($1\times$).

Expression vectors. The *SOX11* open-reading frame clone was purchased from Promega (Tokyo, Japan) and *SOX11* mutants (c.178T>C; p.Ser60Pro and c.347A>G; p.Tyr116Cys) generated by site-directed mutagenesis with the KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan). WT and mutant *SOX11* cDNAs were PCR amplified and cloned into the pEF6/V5-His B mammalian expression vector (Life Technologies) using the In-Fusion PCR Cloning Kit (Clontech Laboratories), and also into the p3xFLAG-CMV-14 mammalian expression vector (Sigma, St Louis, MO). The *GDF5* promoter 5′-flanking sequence ($-448/+319$) was PCR amplified and cloned into the pGL3-basic vector (Promega). All constructs were verified by Sanger sequencing. Human *SOX11* cDNA can be obtained from GenBank/EMBL/DDBJ nucleotide core database under the accession code AB028641.1.

Immunostaining. Mouse neuroblastoma 2A (Neuro-2A) cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–high glucose GlutaMAX supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Life Technologies Co.). Neuro-2A cells were plated into 24-well plates, 24 h before transfection. Each expression construct (200 ng) was transfected into Neuro-2A cells using X-tremeGENE 9 DNA Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) for 15 min at room temperature, and permeabilized in 0.1% Triton X-100/PBS for 5 min at room temperature. C-terminal V5-6xHis-tagged SOX11 proteins were detected using a mouse anti-V5 primary antibody (1:200; Life Technologies Co.) and an Alexa Fluor 546 Goat Anti-Mouse IgG secondary antibody (1:1,000; Life Technologies Co.). Smears were mounted in Vectashield mounting medium with DAPI (Vector Lab., Burlingame, CA). Confocal images were acquired using a Fluoview FV1000-D microscope (Olympus, Tokyo, Japan).

Luciferase assay. HeLa cells were cultured in DMEM-high glucose supplemented with penicillin (50 units ml^{-1}), streptomycin (50 μg ml^{-1}) and 10% FBS. ATDC5 cells were cultured in DMEM/Ham's F-12 (1:1) supplemented with the above antibiotics and 5% FBS. Cells were plated in 24-well plates, 24 h before transfection, and transfections performed using TransIT-LT1 (Takara, Ohtsu, Japan) with pGL3 reporter (500 ng per well), effector (250 ng per well) and pRL-SV40 internal control (6 ng per well) vectors. Twenty-four hours after transfection, cells were harvested and luciferase activities measured using the PicaGene Dual SeaPansy Luminescence Kit (TOYO B-Net, Tokyo, Japan). Production of WT and mutant SOX11 proteins was assessed by immunoblot analysis with monoclonal anti-FLAG M2 HRP antibody (1:3,000; Sigma), following the manufacturer's instructions.

Morpholino and mRNA microinjection. Antisense translation-blocking morpholinos (MOs) for *sox11a*—(5′-CGCTGTTGTCCGTTTGCTGCACCAT-3′), *sox11b*—(5′-CTGTGCTCCGTCTGCTGCACCATGT-3′)[17], *tp53*—(5′-GCGCCATTGCTTTGCAAGAATTG-3′)[18] and standard control—(5′-CCTCTTACCTCAGTTACAATTTATA-3′) MO were obtained from GeneTools (Philomath, OR) and injected (or co-injected) into one- to two-cell-stage embryos at a final concentration of 0.1 or 0.2 mM. In rescue assays, capped human *SOX11* mRNAs transcribed *in vitro* from pEF6/V5-His B constructs were prepared using the mMessage mMachine T7 ULTRA Transcription Kit (Ambion, Carlsbad, CA), following the manufacturer's instructions, and injected into one-cell-stage embryos. For each MO knockdown and rescue experiment, embryos from the same clutch were used as experimental subjects and controls. Approximately 1 ng of capped RNA was injected per embryo. The experiment was authorized by the institutional committee of fish experiments in the National Research Institute of Fisheries Science.

Cell death detection. To detect apoptotic cells in live embryos, embryos at 30 hpf were manually dechorionated and incubated in acridine orange (2 μg ml^{-1} in egg water) at 28 °C for 1 h. After washing with egg water six times for 10 min each, embryos were anaesthetized with tricaine, mounted in 2% methylcellulose and examined by confocal microscopy. Apoptotic cells were also examined by the TUNEL assay, as previously described[26]. Embryos at 30 hpf, were fixed overnight in 4% PFA with PBS at 4 °C and stored in 100% methanol at -20 °C. Samples were incubated in 100% acetone at -20 °C for 20 min. Following fixation, the embryos were rinsed three times with PBS containing 0.1% Tween-20. Samples were then permeabilized by treatment with 0.5% Triton X-100 and 0.1% sodium citrate in PBS for 15 min. Embryos were subjected to the TUNEL assay by using the ApopTag Red *in situ* Apoptosis Detection Kit (Merck KGaA Millipore, Darmstadt, Germany) according to the manufacture's instruction.

Detection and quantitation of visible and fluorescent images. All animals were photographed under the same conditions using a LSM510 confocal microscope (Carl Zeiss, Jena, Germany). In each animal, acridine orange-positive cells were quantitated using a selection tool in Adobe Photoshop, for a colour range chosen by green colour selection of regions showing visually positive acridine orange staining. For analysis of embryos, defined head regions were selected in each embryo. Following pixel selection, a fuzziness setting of 0 was used, and chosen pixel numbers calculated using the image histogram calculation.

Whole-mount immunohistochemistry. For HuC/D staining, embryos at 48 hpf were fixed in 4% PFA overnight at 4 °C and dehydrated in methanol at -20 °C. For acetylated tubulin staining, embryos at 48 hpf were fixed in Dent's fixative (80% methanol and 20% dimethyl sulphoxide) overnight at 4 °C. Embryos were permeabilized with proteinase K followed by postfixation with 4% PFA and washed with PBSTX (PBS containing 0.5% Triton X-100). After treating with 4% normal goat serum (NGS) in PBSTX for 2 h at room temperature, embryos were incubated with mouse anti-HuC/D (1:500, A21271, Life Technologies Co.) or mouse anti-acetylated tubulin (1:1,000, T7451, Sigma) antibodies in 4% NGS/PBSTX overnight at 4 °C. Embryos were washed five times with PBSTX for 10 min each and incubated with goat anti-mouse fluorescein isothiocyanate secondary antibody diluted in 2% NGS/PBSTX for 2 h at room temperature. After washing five times for 10 min each, embryos were mounted in 2% methylcellulose and examined using a Fluoview FV1000-D confocal microscope (Olympus).

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Author contributions

Y.T. and N.Ma. designed and directed the study. Y.T., E.K. and N.Ma. wrote the manuscript. H.O. and S.P. collected samples and provided subjects' clinical information. N.O. evaluated clinical information. Y.T., T.S., S.M., M.N., H.S., S.W., K.-i.Y. and N.Mi. performed exome and Sanger sequencing. E.K., S.Ima. and M.Y. performed zebrafish experiments. I.K. and S.Ike. performed luciferase assays. M.S. and K.O. performed crystal structural analysis. Y.T. and H.K. analysed protein localization.

Additional information

Accession codes: Exome sequence data for CSS patients have been deposited in the Human Genetic Variation Browser under the accession code HGV0000001 (http://www.genome.med.kyoto-u.ac.jp/SnpDB/repository/HGV0000001.html). Access to this data is controlled by the Yokohama City University Data Access Committee.

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ORIGINAL ARTICLE

Predominantly placenta-expressed mRNAs in maternal plasma as predictive markers for twin–twin transfusion syndrome

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ABSTRACT

Objective This study aimed to identify a set of predominantly placental (PP) mRNAs, which are associated with later-developing twin-to-twin transfusion syndrome (TTTS).

Method First, out of 50 PP mRNAs we previously reported, we select target mRNAs that are ordinarily detectable in maternal plasma. Plasma concentrations of these PP mRNAs were measured in monochorionic diamniotic twin (MCDA-T) pregnancies complicated by TTTS later ($n=11$) and in uncomplicated MCDA-T pregnancies ($n=17$). Finally, the diagnostic values of the PP mRNAs in plasma were evaluated.

Results From 50 PP mRNAs, nine [human placental lactogen (*hPL*); pregnancy-specific glycoproteins 2 (*PSG2*); human pregnancy-specific glycoproteins 3 (*PSG3*); *syncytin*; *syncytin 2*; retinoic acid-induced 14; A disintegrin and metalloproteinase domain-containing protein 12 (*ADAM12*); chorionic glycoprotein hormones, alpha polypeptide; and chorionic glycoprotein hormones, and beta polypeptide] were selected as target mRNAs. Changes in six PP mRNAs [increased *hPL*, *PSG2*, and *PSG3* and decreased *syncytin*, *syncytin2*, and *ADAM12*] in maternal plasma were detected in MCDA-T pregnant women who subsequently developed TTTS. Finally, mRNA signatures gave elevated AUCs (*hPL*/*PSG2*: 0.8717; *hPL*/*PSG3*: 0.8449; *hPL*/*ADAM12*: 0.8396) compared with single *hPL* mRNA.

Conclusion Quantitative aberration of plural cell-free PP mRNAs in maternal plasma precedes the appearance of clinically apparent TTTS. This suggests that pathophysiological changes in the placenta are associated with morbid conditions of TTTS. © 2013 John Wiley & Sons, Ltd.

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Conflicts of interest: None declared

INTRODUCTION

Twin–twin transfusion syndrome (TTTS) is a serious complication in monochorionic diamniotic twins (MCDA-T) pregnancies that involves unequal blood flow via placental vascular anastomoses from the donor to the recipient twin. Although placental anastomoses are present in all cases of MCDA-T and both fetuses are genetically identical, TTTS occurs in only 15% of MCDA-T. Much of the pathophysiological basis of TTTS is unclear. Clinically, a staging system based on ultrasound features of TTTS is widely used for its management[1] but not to predict TTTS. New predictive markers are therefore desirable for the early detection and prevention of TTTS.

Recently, placental mRNA, such as human placental lactogen (*hPL*) and other hormones became detectable in maternal plasma; concentrations of each marker were measureable through quantitative real-time reverse transcription polymerase chain reaction (RT-PCR).[2–4] Thus, circulating cell-free mRNA in maternal plasma became an attractive target for non-invasive monitoring of pregnancy disorders.[4,5] Our previous studies indicated that increased levels of cell-free *hPL* mRNA in maternal circulation may be a novel predictive marker for TTTS,[6] although the statistical difference was small and the sample size was too small to give sufficient strength to the analysis. However, a combination of *plural* cell-free placental mRNA markers could effectively predict of TTTS, similar to the situation for tumor markers. Our previous other study to identify predominantly placental (PP) mRNAs, which are only expressed in the placenta, but not in blood cells, identified 50 PP mRNAs by comparing GeneChip signal intensities between placental tissues and corresponding whole blood samples.[7] These 50 PP mRNAs might both help predict and prevent TTTS and further elucidate the pathophysiological condition of TTTS.

In this study, we attempted to identify a set of PP mRNAs, which are associated with later-developing TTTS. We first selected (out of 50 PP mRNAs we had previously reported) PP mRNAs that are constantly detectable in maternal plasma.

Next, to identify PP mRNAs in maternal plasma that predict for TTTS, plasma concentrations of the PP mRNAs were measured in 28 cases of MCDA-T, and differences between MCDA-T pregnancies were complicated by TTTS later ($n=11$), and uncomplicated MCDA-T pregnancies ($n=17$) were evaluated. Finally, we analyzed diagnostic values of PP mRNAs that predicted TTTS.

MATERIALS AND METHODS

Sample collection

The study participants included 28 pregnant women who visited the Obstetrics Clinic of Nagasaki University Hospital at 12 to 21 weeks of gestation for management of their pregnancies with MCDA-T. All of the participants gave written informed consent, and the study was approved by the Research Ethics Committee of Nagasaki University. TTTS was defined as the presence of polyhydramnios (maximum vertical pocket of ≥8 cm) and oligohydramnios (maximum vertical pocket of ≤2 cm).[1] Although none of the 28 cases of MCDA-T were complicated by TTTS at the time of blood sampling, 11 cases subsequently developed TTTS (TTTS group), whereas the remaining 17 cases did not develop TTTS (non-TTTS group). Gestational ages at TTTS diagnosis were 12 to 24 weeks. For a control group, 50 singleton pregnant women without medical complications at similar gestational age were included. The two groups did not significantly differ in population characteristics, including maternal age, number of nulliparous women, and gestational age at the time of sampling (Table 1).

Blood (8 ml) was collected with ethylenediaminetetraacetic acid from the women, and cell-free mRNA was extracted from their plasma (1.6 ml) as described previously.[8]

Quantitative real-time RT-PCR

Because 50 PP mRNAs were identified in our previous study,[7] 50 primer sets for placenta-specific genes (TaqMan® Gene Expression Assays) were purchased from Applied Biosystems (Warrington, UK).[7] One-step quantitative real-time RT-PCR assay was performed using Applied Biosystems (ABI) 7900 T sequence detector (Applied Biosystems) as described previously.[8] RT-PCR amplifications of the placenta mRNA samples were carried out, and the PCR products were sequenced to confirm the accuracy of each gene-specific primer set. As the primer sets for *CSH1* and *HERV-FRD* failed to amplify their exact cDNA sequences, custom primers were made for these genes as described previously.[4,9] The PCR product was cloned into the TOPO II vector (Invitrogen), and the plasmid DNA was extracted to use for a calibration curve. For each mRNA assay, we prepared a calibration curve by tenfold serial dilution of plasmid DNA corresponding to each mRNA sequence from 1.0×10^2 to 1.0×10^8 copies/mL. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect down to 100 copies/mL. Every batch of amplifications included three water blanks as negative controls for each of the reverse transcription and PCR steps. All data were collected and analyzed using an ABI Prism 7900 sequence detector (Applied Biosystems).

Table 1 Clinical characteristics of pregnant women with monochorionic diamniotic twin included in the quantitative analysis of predominantly placental mRNAs in maternal plasma

Characteristics	TTTS group ($n=11$)	Non-TTTS group ($n=17$)	P
Maternal age (years)	29.2 (3.7)°	26.9 (3.8)°	NS
Gestational age at sampling (weeks)	17.0 (6.3)°	15.4 (2.2)°	NS
Parity			NS
Primiparous	6	13	
Multiparous	5	4	

TTTS, twin-to-twin transfusion syndrome; NS, no significant difference between two groups (Student t-test and χ^2 comparisons for continuous and discrete variables, respectively, of TTTS and non-TTTS groups).
$P<0.05$ is considered significant.
°Mean (standard deviation).

Selection criteria for PP mRNAs that are ordinarily found in maternal plasma

Because of the limitation of sample volumes, 50 cases of singleton pregnancy at 12 to 24 weeks of gestation were used to select PP mRNAs that are ordinarily found in maternal plasma. Fifty cases were grouped in five sets of ten cases. Each set was used for measurement of ten kinds of mRNAs. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect down to 100 copies/mL. When cell-free PP mRNA in plasma was consistently detectable (>100 copies/mL) in all of the ten cases, these mRNAs were selected as the target mRNAs in this study.

Statistical analysis

Patient backgrounds were compared by Student's t-test and Pearson's χ^2-test for continuous and discrete variables, respectively, of TTTS and non-TTTS groups. Absolute quantification data were analyzed with SDS 2.3 software (Applied Biosystems). Cell-free plasma concentrations of PP mRNAs in the TTTS and non-TTTS groups were converted into multiples of the median of the controls adjusted for gestational age, as described previously.[6,10] Differences between the two groups were evaluated with Mann–Whitney U-test, and differences between the three groups were examined with the Kruskal–Wallis test. Statistical analyses were performed with SPSS version 19 (IBM Japan, Tokyo, Japan). To determine the use of PP mRNAs to classify TTTS and non-TTTS later, receiver operating characteristic (ROC) curves were plotted with an R package, pROC.[10] To develop mRNA signatures that most accurately distinguished between patients who developed TTTS later and those who did not, we used a multivariate logistic regression model. The obtained regression models were evaluated with Wald tests. Statistical analyses used R (R Core Team, Vienna, Austria). $P<0.05$ was considered significant.

RESULTS

PP mRNAs that could ordinarily be measured in maternal plasma

Of 50 PP mRNAs, nine mRNA [*hPL*; human pregnancy-specific glycoproteins 2 (*PSG2*); human pregnancy-specific glycoproteins 3 (*PSG3*); *syncytin*; *syncytin 2*; retinoic acid-induced 14 (*RAI14*); A

disintegrin and metalloproteinase domain-containing protein 12 (*ADAM12*); chorionic glycoprotein hormones, alpha polypeptide (*CGA*); and chorionic glycoprotein hormones, and beta polypeptide (*CGB*)] were detectable in all ten singleton pregnancy plasma samples; these were selected as target mRNAs in this study.

Identification of cell-free PP mRNAs, which are associated with later-developing TTTS

Circulating levels of the nine PP mRNAs in plasma from the cases of MCDA-T and the control cases were measured by quantitative RT-PCR. The quantitative aberrations of six cell-free PP mRNAs (increased *hPL*, *PSG2*, and *PSG3* and decreased *syncytin*, *syncytin2*, and *ADAM12*) in maternal plasma were detected in MCDA-T pregnant women who subsequently developed of TTTS (Kruskal–Wallis test, *P* values were 0.005, 0.001, 0.002, 0.044, 0.027, and 0.012, respectively; Table 2), whereas there was no significant differences of three cell-free PP mRNAs (*RAI14*, *CGA*, and *CGB*) levels and cell-free *GAPDH* mRNA level in maternal plasma between TTTS, non-TTTS, and control groups (Table 2). The circulating levels of three PP mRNAs (*hPL*, *PSG2*, and *PSG3*) were significantly higher in plasmas from those who developed TTTS later than in plasmas from patients who did not (non-TTTS) (Mann–Whitney *U*-test, *P* values were 0.014, 0.007, and 0.003, respectively; Table 2, Figure 1). Circulating levels of three PP mRNAs (*syncytin*, *syncytin2*, and *ADAM12*) were significantly lower in plasmas from those who developed TTTS later than in plasmas from non-TTTS patients (Mann–Whitney *U*-test, *P* values were 0.041, 0.029, and 0.003, respectively; Table 2, Figure 1). Plasma levels of three PP mRNAs (*RAI14*, *CGA*, and *CGB*) and *GAPDH* mRNA did not significantly differ between the two groups (Mann–Whitney *U*-test, *P* values were 0.525, 0.655, 0.944, and 0.778, respectively; Table 2, Figure 1).

Diagnostic value of PP mRNAs in plasma

The ROC curves to discriminate the TTTS group from the non-TTTS group were constructed on the basis of PP mRNAs in plasma samples (TTTS, $n=11$; non-TTTS, $n=17$). Analysis of ROCs revealed high area under the curve (AUC) values for each

Table 2 Circulating levels of predominantly placenta-expressed miRNAs in plasma samples from patients with monochorionic diamniotic twin pregnancies who developed twin-to-twin transfusion syndrome (TTTS) later (TTTS group) and from such patients who did not develop TTTS (non-TTTS group) and singleton pregnant women without medical complications (control group)

mRNA	TTTS group ($n=11$)	non-TTTS group ($n=17$)	Control group ($n=10$)	*P*
hPL	1.78 (1.12–2.80)	1.15 (0.89–1.87)	1.0 (0.68–2.56)	0.005
PSG2	2.60 (1.28–4.04)	1.37 (0.58–3.20)	1.0 (0.52–2.74)	0.001
PSG3	3.13 (0.70–12.3)	0.99 (0.42–4.23)	1.0 (0.39–2.86)	0.002
Syncytin	0.46 (0.22–1.89)	1.12 (0.21–3.43)	1.0 (0.21–3.18)	0.044
Syncytin2	0.69 (0.48–0.99)	0.95 (0.46–3.53)	1.0 (0.42–3.37)	0.027
ADAM12	0.49 (0.31–1.39)	1.18 (0.31–2.31)	1.0 (0.25–1.46)	0.012
RAI14	1.13 (0.50–1.54)	1.21 (0.31–2.31)	1.0 (0.35–2.06)	NS
CGA	1.05 (0.36–1.49)	1.19 (0.29–1.68)	1.0 (0.33–1.61)	NS
CGB	0.95 (0.41–1.34)	0.92 (0.27–1.76)	1.0 (0.39–1.76)	NS
GAPDH	1.10 (0.51–2.17)	1.01 (0.49–2.62)	1.0 (0.47–2.17)	NS

NS, not significant.

Expression levels are shown as multiple of median, with median (minimum–maximum range). Significant differences between groups were analyzed by Kruskal–Wallis test. $P<0.05$ is considered significant.

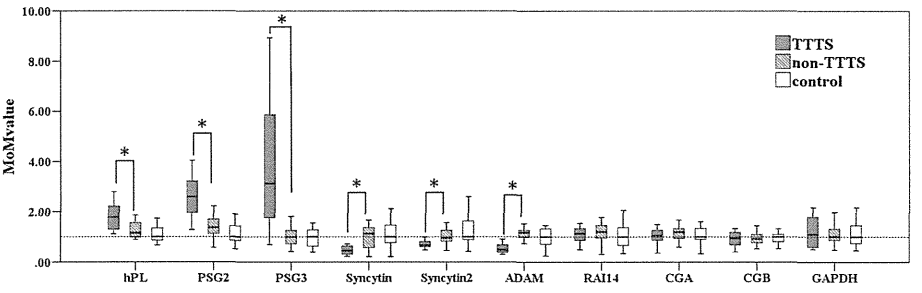

Figure 1 Circulating levels of predominantly placental mRNAs in maternal plasma samples from twin-to-twin transfusion syndrome (TTTS) group and non-TTTS group. Circulating levels of cell-free mRNA in maternal plasma were expressed as multiple of median (MoM) values. Gray bars indicate data from cases that later developed TTTS; shaded bars indicate the data from cases that did not develop TTTS (non-TTTS); white bars indicate the data from singleton pregnant women (control). *Significant difference ($P<0.05$)

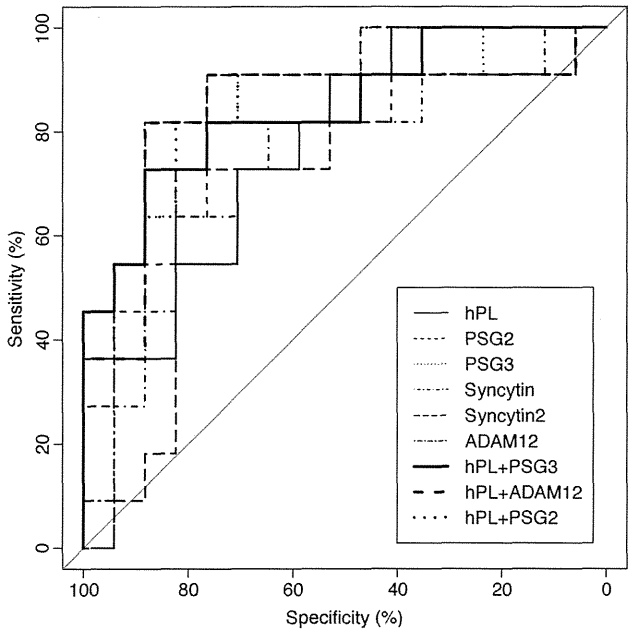

Figure 2 Receiver operating characteristic analysis of plasma mRNA profiles to discriminate cases of twin-to-twin transfusion syndrome (TTTS) later from cases of non-TTTS later. Analysis of receiver operating characteristics revealed high AUC values for each predominantly placental mRNA in plasma (TTTS: $n=11$; non-TTTS: $n=17$). The mRNA signatures consisting of two predominantly placental mRNAs yielded elevated AUCs compared with single *hPL* mRNAs

PP mRNA in plasma (Figure 2): *hPL*, 0.7807 (95% CI: 0.6083–0.9532), *PSG2*: 0.8075 (95% CI: 0.6383–0.9767), *PSG3*: 0.8396 (95% CI: 0.6785–1.0), *syncytin*: 0.0.7326 (95% CI: 0.5291–0.9362), *syncytin2*: 0.7487 (95% CI: 0.559–0.9383), and *ADAM12*: 0.8342 (95% CI: 0.648–1.0).[11]

The mRNA signatures consisting of two PP mRNAs yielded elevated AUCs in comparison to single *hPL* mRNAs (Figure 2): *hPL/PSG2*: 0.8717 (95% CI: 0.7400–1.0, $P=0.1124$, Wald test), *hPL/PSG3*: 0.8449 (95% CI: 0.6893–1.0, $P=0.0929$, Wald test), and *hPL/ADAM12*: 0.8396 (95% CI: 0.6579–1.0, $P=0.9571$, Wald test).

DISCUSSION

In this study, we found that changes in plasma concentrations of plural PP mRNAs were detectable before the appearance of clinically apparent TTTS. In addition, we evaluated the diagnostic significance of a set of PP mRNAs in maternal plasma.

As non-invasive potential predictive markers for patients who develop TTTS later, six PP mRNAs in maternal plasma were identified – three that increased (*hPL*, *PSG2*, and *PSG3*) and three that decreased (*syncytin*, *syncytin2*, and *ADAM12*). *PSG2*, *hPL*, and *PSG3* are synthesized in large amounts by the placental syncytiotrophoblast and are released into the maternal circulation during pregnancy.[12,13] *Syncytin1* and *syncytin2*, which are placenta-specific env genes of endogenous retroviruses, have fusogenic activity in promoting the formation of multinucleated syncytiotrophoblast cells in the placenta.[14,15] *ADAM12* is one of the most promising protein candidates for participation, as well as *syncytin*, in the process of trophoblast fusion in the placenta.[15,16] The current study found that changes in plasma concentrations of PP mRNAs, which are important molecules for placentation and maintenance of pregnancy, preceded the appearance of clinically apparent TTTS. This suggests that abnormal placentation and abnormalities in the placenta are associated with the pathophysiological basis of TTTS.

Although a staging system based on the ultrasound features of TTTS is widely used for its management,[1] this system is not generally used to predict TTTS.

These plasma-based biomarkers may lead to development of a non-invasive means of predicting later TTTS. Expression patterns for mRNA are known to be aberrant in pregnancy-related diseases (e.g. preeclampsia, placenta previa)[2,3,7,8]; placenta-derived mRNAs in circulation may be stored in microvesicles.[17] Additionally, cell-free mRNAs are remarkably stable in plasma.[2–4] Our observations suggest that PP mRNAs in plasma might serve as a non-invasive biomarker for early detection of TTTS later. Although the source of plasma PP mRNAs has not been determined, they might derive from exosomes shed from apoptotic or broken placental cells.[8,17,18] Therefore, although this is a matter of speculation, abnormal placentation caused by a decreased level of PP mRNAs (*syncytin*, *syncytin2*, and *ADAM12*) at the early stage of pregnancy may lead to increased levels of circulating microparticles, which include placental molecule (*hPL*, *PSG2*, and *PSG3*) synthesized by syncytiotrophoblast, into maternal plasma.

The specific conditions that regulate levels of the maternal plasma mRNA in the TTTS group remain unknown; possibly, unapparent pathophysiological changes had already occurred in the women who subsequently developed TTTS. ROC curve analysis revealed that six single-regulated PP mRNAs in plasma samples from MCDA-T pregnancies could distinguish between TTTS and non-TTTS pregnancies later, yielding high AUCs (Figure 2). The mRNA signatures consisting of two PP mRNAs that yield elevated AUCs compared with single *hPL* mRNAs, although the PP mRNA signatures and a single *hPL* mRNA did not significantly differ. The known predictive findings (e.g. growth discordancy, nuchal translucency) by ultrasonographic examination are only detectable in a small portion of TTTS cases,[19] although they can be measured simply and non-invasively. In contrast, expression profiles of PP mRNAs in maternal plasma differ between MCDA-T pregnancies that are, and are not, complicated by TTTS later, suggesting that PP mRNAs signatures in maternal plasma may be used as additional markers for TTTS prediction in MCDA-T pregnancies. As the pathogenesis of TTTS may be associated with multiple factors, TTTS could be regulated by a large variety of genes. Furthermore, another study demonstrated a significant difference in cell-free vascular endothelial growth factor A, endoglin, and angiopoietin-2 mRNA levels in maternal plasma between MCDA-T pregnancies complicated by TTTS and uncomplicated MCDA-T pregnancies.[20] The aforementioned observations suggest that placental factor and other factors are associated with TTTS pathogenesis of TTTS. Therefore, as with markers for

malignant tumors, a combination of plural mRNA markers (placental factor, PP mRNAs; other factors, vascular endothelial growth factor A, *endoglin*, and angiopoietin-2 mRNAs, etc.) and ultrasonographic findings (e.g. growth discordancy, nuchal translucency) may predict MCDA-T pregnancies later complicated by TTTS more efficiently, compared with a single PP mRNA marker.[20]

In conclusion, a cohort of MCDA-T pregnancies, in which some were complicated by TTTS later, showed that changes in plural cell-free PP mRNA levels in maternal plasma preceded the appearance of clinically apparent TTTS. This was a preliminary study with a small sample size. Therefore, a large prospective study measuring placental factors in MCDA-T pregnancies would help to confirm the diagnostic significance of a set of cell-free PP mRNAs as a predictive test for TTTS. Future studies regarding the biological pathway of PP mRNAs in maternal plasma as predictors of TTTS may help elucidate the molecular pathogenesis of TTTS.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Predominantly placental (PP) mRNAs, for example, human placental lactogen (*hPL*), in maternal plasma are measurable by quantitative reverse transcription polymerase chain reaction.
- Increased cell-free *hPL* mRNA in maternal plasma may predict twin-to-twin transfusion syndrome (TTTS).
- We found 50 PP mRNAs using microarray analysis.

WHAT DOES THIS STUDY ADD?

- In addition to *hPL* mRNA, quantitative aberration of plural PP mRNAs precedes the appearance of clinically apparent TTTS.
- We evaluated these PP mRNAs in maternal plasma as TTTS predictors.

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ORIGINAL ARTICLE

Single human papillomavirus 16 or 52 infection and later cytological findings in Japanese women with NILM or ASC-US

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The relationship between oncogenic human papillomavirus (HPV) infection and later cytological findings in the uterine cervix is unknown in women who were negative for intraepithelial lesion and malignancy (NILM) or atypical squamous cells of undetermined significance (ASC-US). This was investigated in this study in a Japanese population to determine the clinical utility of oncogenic (HPV) genotyping. The relative risk of progressive cytological findings 2 years after identification of oncogenic HPV infection was higher than in cases of non-oncogenic HPV infection (relative risk 3.827; 95% confidence interval (CI): 1.282–11.422), as well as in cases of negative HPV infection (relative risk 2.124; 95% CI: 1.451–3.110). Moreover, the relative risk of progression of cytological findings 2 years later in cases of HPV-16 infection was higher than in cases of HPV-52 infection (relative risk 2.094; 95% CI: 1.005–3.935). Therefore, the initial HPV-DNA genotype may be a potential predictive marker of later progression of cytological findings in the uterine cervix in cases of NILM or ASC-US.
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Keywords: cytological findings; genotype; human papillomavirus; oncogenic HPV-16; oncogenic HPV-52; uterine cervical cancer

INTRODUCTION

Persistent infections with oncogenic human papillomaviruses (HPVs), including 16 different HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82), are recognized as a major risk factor for the development of cervical cancer.[1] HPV-16 and HPV-18 infection accounts for approximately 70% of cancers and 50% of high-grade cervical intraepithelial neoplasia.[2] Although the risk of cervical cancer may be an order of magnitude higher for HPV-16 infection than other oncogenic HPV types, our previous studies showed that HPV-52 was a more common genotype in Nagasaki, Japan, compared with the distribution of high-risk HPV genotypes in other countries.[3,4] In addition, HPV-52 was the most common genotype among HPV-infected pregnant Japanese women. The second most common genotype was HPV-16, and these two genotypes collectively accounted for around 60% of HPV-positive pregnant women.[4] Hence, geographic variations in HPV-type distributions should be an important consideration.

The screening of high-grade squamous intraepithelial lesions (HSILs) and cervical cancer currently involves the detection of oncogenic HPV by cervicovaginal Pap smears,[5] and it is both important and necessary to clarify the association between the individual oncogenic HPV genotypes in each region and the progression of later cytopathological findings in the uterine cervix. When women are screened using cervicovaginal Pap smears, most are diagnosed with negative for intraepithelial lesion or malignancy (NILM) or atypical squamous cells of undetermined significance (ASC-US). ASC-US consists of various cytological abnormalities, which may contain low-grade squamous intraepithelial lesions or HSILs of the uterine cervix. Therefore, testing for oncogenic HPV is now routinely carried out in both Japan and the United States for managing women with ASC-US. However, the clinical usefulness of oncogenic HPV testing is limited by the fact that oncogenic HPV infections are relatively common among women without cervical intraepithelial neoplasia (CIN) or cancer. Also, in the United States, the clinical usefulness of HPV-16 and/or HPV-18 genotyping to triage oncogenic HPV-positive women with NILM aged 30 years and above has been recently recognized in clinical management guidelines; HPV-16-positive or HPV-18-positive women with NILM are referred for colposcopy, whereas other oncogenic HPV-positive women with NILM are recommended to repeat the cervical cytology and oncogenic HPV testing in 12 months.[6] Nevertheless, the data supporting the use of HPV genotyping in this manner are relatively

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limited. Moreover in clinical management, it is not obvious how oncogenic HPV-positive women with NILM or ASC-US cytology should be managed in primary cervical cancer screening, or how the presence of oncogenic HPV infection affects later cytological findings in Japanese women with a cytological diagnosis of NILM or ASC-US at first admission.

Therefore, this study aimed to understand the clinical utility of oncogenic HPV genotyping in Japanese women by investigating the relationship between oncogenic HPV infection in a Japanese population and cytological changes in the uterine cervix. Subsequently, we investigated how a single HPV-16 or HPV-52 infection affects the cytological findings in Japanese women with NILM or ASC-US 2 years later.

MATERIALS AND METHODS

Patients

Women admitted to five hospitals in Nagasaki Prefecture, Japan, between 2007 and 2011 as a pre-vaccination population for the HPV vaccine[3,4] were informed of the purpose of the study and gave their consent for participation. We identified 319 cases with a cytological diagnosis of NILM or ASC-US at first admission, and followed them up by cervicovaginal Pap smears and HPV genotyping over the next 2 years. The study protocol was approved by the ethical review board of Nagasaki University and the other hospitals involved.

Sample collection and cytological diagnoses

Specimens were collected using a Cervex Brush (Rovers Medical Devices, Oss, The Netherlands) and suspended in 10 ml of SurePath preservative fluid (Becton Dickinson, Franklin Lakes, NJ, USA). Samples from the same vial were used for cytological testing with the Bethesda III system (2001) and for HPV genotype testing.[3,4] Cervical specimens for cytology and HPV genotyping were obtained at each visit from participants who received regular follow-up examinations. To minimize the possibility of diagnostic variations, cytologic diagnoses of the specimens were performed by the same experienced cytoscreener and a qualified medical doctor in a commercial laboratory (SRL, Tokyo, Japan), who were blinded to the results of the HPV genotyping test. Cervical cytological findings were reported as NILM, ASC-US, low-grade squamous intraepithelial lesion, atypical cells—cannot exclude HSILs (ASC-H) and HSIL. Regarding cervical cytological findings determined 2 years after first admission, cases showing progression (changing from 'NILM' to 'ASC-US or worse cytological findings', or from 'ASC-US' to 'low-grade squamous intraepithelial lesion or worse cytological findings') were defined as the progression group, and cases showing no change or regression (from 'NILM' to 'NILM', or from 'ASC-US' to 'ASC-US or NILM') were defined as the non-progression group. Colposcopy was performed in the cases diagnosed as ASC-US with oncogenic HPV infections (data not shown).

HPV genotyping test

Genotyping of HPV DNA in SurePath preservative fluid was carried out after preparing glass slides using the Linear Array HPV Genotyping Test Kit (Roche Molecular Systems, Indianapolis, IN, USA). PGMY09/PGMY11 primers[7] amplified the L1 conserved region by polymerase chain reaction, and hybridization of the HPV amplicon was performed using an array of oligonucleotide probes that allowed the independent identification of individual HPV genotypes. This kit can detect the following 37 HPV genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108 (89). For consistency with previous studies, 16 HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) were considered to be high risk as they had previously been related to cervical cancer.[1,8,9]

Statistical analysis

Patient backgrounds were compared between the progression and non-progression groups using Student's *t*-tests and χ^2 tests for continuous and discrete variables, respectively. Differences in oncogenic HPV genotyping between the two groups were evaluated using relative risk. Statistical analyses were performed with SPSS software version 19 (IBM Japan, Tokyo, Japan). Significant differences were defined as $P<0.05$.

RESULTS

Study population backgrounds

Of the 319 Japanese women (253 cases of NILM and 66 cases of ASC-US) originally studied, HPV genotyping showed that 178 (159 cases of NILM and 19 cases of ASC-US) had no HPV infection, 26 (17 cases of NILM and 9 cases of ASC-US) had only a non-oncogenic HPV infection, 38 (25 cases of NILM and 13 cases of ASC-US) had multiple oncogenic HPV infections, and 77 (52 cases of NILM and 25 cases of ASC-US) had a single oncogenic HPV infection (Figure 1). The 77 cases with a single oncogenic HPV infection included HPV-16 ($n=20$; 13 cases of NILM and 7 cases of ASC-US), HPV-18 (1 case of NILM), HPV-31 ($n=7$; 6 cases of NILM and 1 case of ASC-US), HPV-35 (2 cases of ASC-US), HPV-39 (1 case of ASC-US), HPV-51 ($n=3$; 1 case of NILM and 2 cases of ASC-US), HPV-52 ($n=29$; 22 cases of NILM and 7 cases of ASC-US), HPV-58 ($n=11$; 6 cases of NILM and 5 cases of ASC-US), HPV-59 (2 cases of NILM) and HPV-68 (1 case of NILM) (Figure 1). Of the 25 cases of ASC-US with a single oncogenic HPV infection, 12 were followed up with HPV genotyping. Of the 12 cases of ASC-US with a single oncogenic HPV infection at first admission, persistent infection of oncogenic HPV was detected 2 years later in 4 (33.3%; 3 cases showing a cytological 'progression', and 1 case showing a cytological 'non-progression'), while oncogenic HPV had disappeared in the remaining 8 cases (66.7%, all 8 cases showing a cytological 'non-progression' 2 years later; $P=0.005$, χ^2 test).

There was no significant difference in patient background between the single oncogenic HPV infection and HPV-negative groups (Table 1), the single oncogenic HPV infection and single non-oncogenic HPV infection groups (Table 1), or between the HPV-16 infection and HPV-52 infection groups (Table 2). There was no significant difference in age distributions between the progression and the non-progression groups (Table 3).

Relationship between oncogenic HPV infection and the progression of cytological findings in Japanese women with NILM or ASC-US

Of the 77 cases with a single oncogenic HPV infection, 30 (38.96%) were classified in the progression group, whereas 47 (61.04%) were in the non-progression group. This compares with 37 (20.79%) and 141 (79.21%), respectively, for the 178 cases of negative HPV infection, and 3 (11.54%) and 23 (88.46%), respectively, for the 26 cases of non-oncogenic HPV infection. The relative risk of progression of cytological findings 2 years later in cases of oncogenic HPV infection was higher than in cases of non-oncogenic HPV infection, as well as in cases of negative HPV infection (Table 4). However, the relative risk of progression of cytological findings 2 years later in cases of non-oncogenic HPV infection was not higher than in cases of negative HPV infection (Table 4).

Association between single HPV-16 or HPV-52 infection and later cytological findings in Japanese women with NILM or ASC-US

In the 20 cases of single HPV-16 infection (HPV-16 group), 13 (65%) were classified in the progression group, whereas 7 (35%) were in the

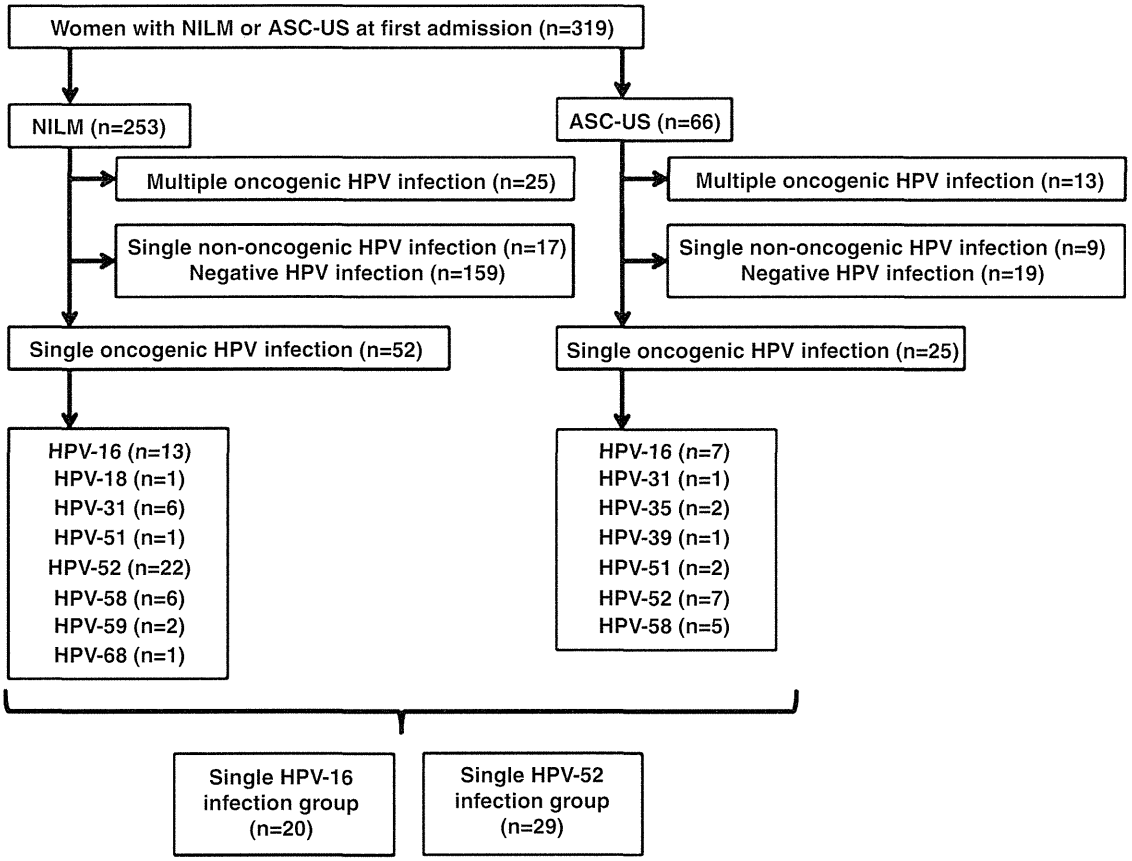

Figure 1 Results of HPV genotyping in women with NILM or ASC-US at first admission. ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; NILM, negative for intraepithelial lesion and malignancy.

Table 1 Patient background in cases of single oncogenic HPV, non-oncogenic HPV or negative HPV infections

	Results of HPV test			*P-value*	
	A	*B*	*C*		
	Oncogenic HPV group (n = *77*)	*Non-oncogenic HPV group* (n = *26*)	*Negative HPV group* (n = *178*)	*A* vs *B*	*A* vs *C*
Age at first sampling (years)[a]	42.58 (11.78)	40.27 (14.18)	41.95 (11.03)	0.232	0.876
Smoking[b]	12 (15.6%)	6 (23.1%)	21 (11.8%)	0.384	0.408
Use of oral contraceptive[b]	3 (3.9%)	0 (0.0%)	6 (3.4%)	0.307	0.835
Parity[b]					
Nulliparous	24 (31.2%)	5 (19.2%)	60 (33.7%)	0.495	0.924
Primiparous	14 (18.2%)	6 (23.1%)	31 (17.4%)		
Multiparous	39 (50.6%)	15 (57.7%)	87 (48.9%)		
Body mass index ($kg\,m^{-2}$)[a]	21.32 (2.47)	21.17 (2.34)	21.27 (2.76)	0.963	0.727

Abbreviation: HPV, human papillomavirus.
[a]Mean (s.d.). Student's *t*-test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.
[b]Number of cases (percentage). χ^2 test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.

non-progression group. This compares with 9 (31.03%) and 20 (68.97%), respectively, for the 29 cases of single HPV-52 infection (HPV-52 group). The relative risk of progression of cytological findings 2 years later in cases of HPV-16 infection was higher than in cases of HPV-52 infection (Table 5).

DISCUSSION

In this study, we investigated the association between common oncogenic HPV infection in a Japanese population and the progression of cytological findings 2 years later in Japanese women with NILM or ASC-US.

Matsumoto *et al.*[10] previously reported that in Japanese women with cytological low-grade squamous intraepithelial lesion and histological CIN grade 1–2 lesions, the oncogenic HPV infection group showed a greater tendency to develop CIN3 within 5 years than the non-oncogenic HPV infection group.[1] Consistent with these results, we confirmed that infection with common oncogenic HPV in Japan poses a higher risk for the progression of cytological findings

in the uterine cervix 2 years later than non-oncogenic HPV infection or no HPV infection.

As our study cohort was a pre-vaccination population; there is no influence of the HPV vaccine on our observations. Although more spontaneous regression of HPV infection usually occurs among young women, there was no significant difference in age distributions between the progression and the non-progression groups in this study. Therefore, we were able to investigate how the HPV genotype affects later cytological findings in Japanese women with NILM or ASC-US. Our investigation suggests that Japanese women with oncogenic HPV infections should undergo periodic checkups for uterine cervical cancer over the course of 2 years from initial identification of infection. In a previous study by Dufresne *et al.*,[11] persistent infection of oncogenic HPV was detected in 33 (67.6%) of 34 cases of ASC-US with oncogenic HPV infections, while in the remaining 11 cases (32.4%), 6 months later oncogenic HPV had disappeared and there was no sign of CIN2/3. In our study, among the 12 single oncogenic HPV-positive women diagnosed with ASC-US at first admission, persistent infection of oncogenic HPV was detected in four (33.3%), while oncogenic HPV had disappeared in the remaining eight cases (66.7%) 2 years later and there was no sign of cytological progression in these eight cases 2 years later. These results confirm that, as time passes, oncogenic HPV can disappear spontaneously in women with ASC-US, and that the negative predictive value of the HPV test may help to reduce the number of unnecessary colposcopies carried out in women with ASC-US.[11]

Khan *et al.*[12] reported that among the cases of NILM with HPV-16, the 10-year cumulative rate of CIN3 or cancer was 17% and the 2-year cumulative incidence rate of CIN3 or cancer was 9.8%. This is similar to our results, which showed that 3 (15%) of 20 single HPV-16-positive cases of 'NILM or ASC-US' at first admission were diagnosed with ASC-H or HSIL by cytological testing 2 years later (data not shown). Recently, the ATHENA (Addressing the Need for Advanced HPV Diagnostics) study reported that, among cases of NILM or ASC-US, the estimated absolute risk of CIN2 or worse in cases of HPV-16 and/or HPV-18 was higher than in cases of other oncogenic HPV.[13–15] Our result is consistent with these studies that showed HPV-16 is associated with a higher risk of cervical cancer onset than oncogenic HPV genotypes in other regions.[16] We clarified that a single HPV-16 infection of women with 'NILM or ASC-US'

Table 2 Patient backgrounds in cases of single HPV 16 or single HPV 52 infections

	Single HPV-16 infection group (n = 20)	*Single HPV-52 infection group (n = 29)*	P-*value*
Age at first sampling (years)[a]	43.60 (15.99)	40.83 (10.01)	0.768
Smoking[a]	5 (25%)	4 (13.8%)	0.319
Use of oral contraceptive[b]	1 (5%)	0 (0%)	0.224
Parity[b]			
Nulliparous	7 (35%)	11 (37.9%)	0.381
Primiparous	5 (25%)	3 (10.3%)	
Multiparous	8 (40%)	15 (51.7%)	
Body mass index ($kg\,m^{-2}$)[a]	21.84 (3.27)	21.42 (2.15)	0.935

Abbreviation: HPV, human papillomavirus.
[a]Mean (standard deviation). Student's *t*-test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.
[b]Number of cases (percentage). χ^2 Test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.

Table 3 Age distributions between the progression and the non-progression groups

Age (years)	*Progression group*	*Non-progression group*	P-*value*[a]
20–29	4	35	0.06
30–39	24	72	
⩾40	42	104	
Total cases	70	211	

[a]Pearson's χ^2 test was used to analyze differences in age distributions between groups. Statistical significance was defined as $P<0.05$.

Table 5 Association between common oncogenic HPV genotype in Japan and progression of cytological findings 2 years later

	Results of HPV test			
	A Single HPV-16 infection	*B Single HPV-52 infection*	*Total cases*	*Relative risk (95% confidence interval) A vs B*
Progression group	13	9	22	2.094 (1.005–3.935)
Non-progression group	7	20	27	
Total cases	20	29	49	

Abbreviation: HPV, human papillomavirus.

Table 4 Association between HPV infection and progression of cytological findings 2 years later

	Results of HPV test				*Relative risk (95% confidence interval)*		
	A Single oncogenic HPV infection	*B Single non-oncogenic HPV infection*	*C Negative HPV*	*Total cases*	*A vs B*	*A vs C*	*B vs C*
Progression group	30	3	37	70	3.376 (1.282–11.422)	1.874 (1.451–3.110)	2.012 (0.573–7.067)
Non-progression group	47	23	141	211			
Total cases	77	26	178	281			

Abbreviation: HPV, human papillomavirus.

cytology is associated with a higher risk of progression of cytological findings 2 years later compared with a single HPV-52 infection, supporting the recommendation of colposcopy for HPV-16 genotyping of women with NILM or ASC-US cytology.[6]

According to the Cochrane Database of Systematic Reviews, HPV-triage with hybrid capture 2 is recommended to triage women with ASC-US because of its greater accuracy (significantly higher sensitivity, and similar specificity) compared with repeat cytology.[17] Regarding uterine cervical cancer in Japanese women, the genetics of HPV infection (oncogenic HPV genotype and oncogenic HPV viral loads) is not sufficient to cause CIN3/cervical cancer, and host genetic factors are also likely to contribute to cervical cancer pathogenesis.[2,4,5,8,18–22] Further study focused on cancer genetics in both the HPV virus and host may enable the development of a comprehensive system of early detection and prevention of uterine cervical cancer.

In conclusion, for the first time, we confirmed that common oncogenic HPV infection is associated with a higher risk of progression of cytological findings 2 years later in Japanese women with NILM or ASC-US; of these infections, HPV-16 was found to have a higher risk of progressive cytological findings than HPV-52. Therefore, the initial HPV-DNA genotype may be a potential predictive marker of later progression of cytological findings in the uterine cervix in Japanese women with NILM or ASC-US.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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