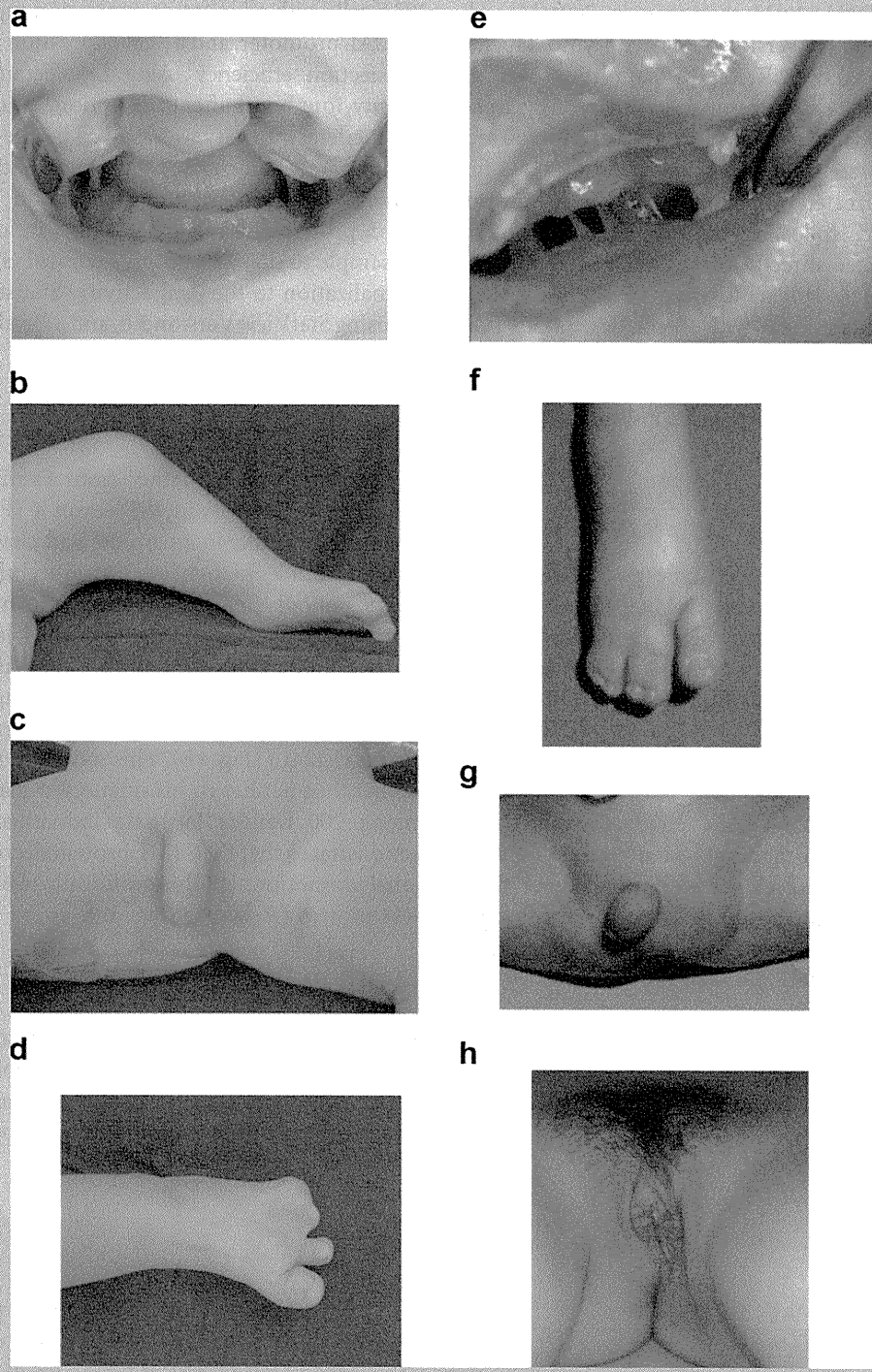

FIG. 2. Phenotypic appearance of the proband (a–d) from Family 1, and the proband (e–g) and his mother (h) from Family 2. a: Bilateral cleft lip and palate, lower lip pits, syngnathia; (b) popliteal web; (c) absent scrotum; (d) syndactyly; (e) syngnathia; (f) syndactyly; (g) unclear scrotum; (h) hypoplastic labia majora of mother.

proband, his parents and maternal grandmother in Family 2. All experimental procedures were approved by the Committees for the Ethical Issues on Human Genome and Gene Analysis in Aichi-Gakuin University and in Nagasaki University.

IRF6 Mutation Search

Screening for mutations in *IRF6* was performed in individuals from the two families. All exons and exon–intron boundaries of *IRF6* were amplified by PCR using primer pairs designed from the

genomic sequence. PCR was performed in a 10 μl mixture containing 5 ng of genomic DNA, 1 μM each primer, 200 μM each dNTPs, 0.3 U TaKaRa ExTaq HS version (TaKaRa, Kyoto, Japan), and 10× PCR buffer supplied by TaKaRa. PCR conditions were as follows; initial incubation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 7 min. PCR products were treated with ExoSAP-IT (GE Healthcare, Buckinghamshire, England) following the supplier's instruction manual, and then sequenced directly using BigDye Terminator ver.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequenced samples were purified with Sephadex G-50 (GE Healthcare) and run on an automated sequencer Model 3100 (Applied Biosystems). Sequence electropherograms were aligned using AutoAssembler software v2.1 (Applied Biosystems) to find a base alteration by visual inspection.

Construction of Plasmid Expressing *IRF6*

We generated plasmids expressing the wild-type (pS424) or mutant (pL424) *IRF6* by site-directed mutagenesis using the Genetailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA). To express a GAL4-IRF6 fusion protein, we fused the DNA sequence for IRF6 C-terminus portions (amino acid positions 114 to 467) to GAL4 DNA binding domain (DBD). We used pBIND-DEST expressing vector containing the GAL4-DNA binding domain in Gateway system (Invitrogen). Thus, each of the recombinant plasmids (pS424 and pL424) contained the sequence for the GAL4 -DBD and the IRF6 interaction domain with Ser or Leu at the 424th amino acid residue (Fig. 3a). All clones were verified for their integrity by sequencing, and their expressions were verified by western blot analysis with a polyclonal anti-IRF6 antibody (Active Motif, Carlsbad, CA) (data not shown).

Cell Culture, Transfection, and Luciferase Assay

We cultured 293T cells in Dulbecco's MEM (Sigma-Aldrich, St. Louis, MO) containing 10% FBS at 37°C in 5% CO_2. We transiently transfected expression plasmids (pS424 or pL424) to 293T cells with a luciferase-expression reporter plasmid driven by the GAL promoter, and a βgal-expression plasmid to normalize the transfection efficiency using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, soluble protein was extracted and luciferase activity was assayed by the Luciferase Assay System (Promega, Madison, WI) using Galacto-Light Plus (Applied Biosystems). Transcriptional activation by the GAL4DBD-IRF6 fusion protein was calculated as the ratio of luciferase activity in the sample to that of a positive control GAL4-DBD plasmid, after normalization to the βgal activity. Statistical analysis was carried out using StatView Version 5.0, and a *P*-value <0.05 was considered to be statistically significant.

RESULTS

The proband and his father from Family 1 showed a missense mutation, c.251G>T, in *IRF6*-exon 4 (Fig. 1A). This nucleotide substitution results in an amino acid change in the DNA binding domain from a polar charged basic arginine residue into a hydrophobic nonpolar leucine (R84L). No such mutation was present in either the mother or among 90 healthy Japanese individuals.

In the proband from Family 2, a missense mutation, c.1271C>T, was found in the last exon (exon 9) giving a predicted amino acid change from a polar uncharged serine to a hydrophobic nonpolar leucine (S424L) (Fig. 1B). This mutation was found in his affected mother, but neither in his unaffected maternal grandmother nor among 200 healthy Japanese individuals. The luciferase assay demonstrated that the S424L protein decreased the *IRF6* transcriptional promotion activity significantly to 6% of that of the wild-type S424 protein ($P < 0.05$) (Fig. 3b).

DISCUSSION

We have identified two mutations, R84L and S424L, in two Japanese families affected by PPS. Both mutations were hitherto undescribed, although R84H in two families and R84C in five families have previously been reported to be associated with PPS [Kondo

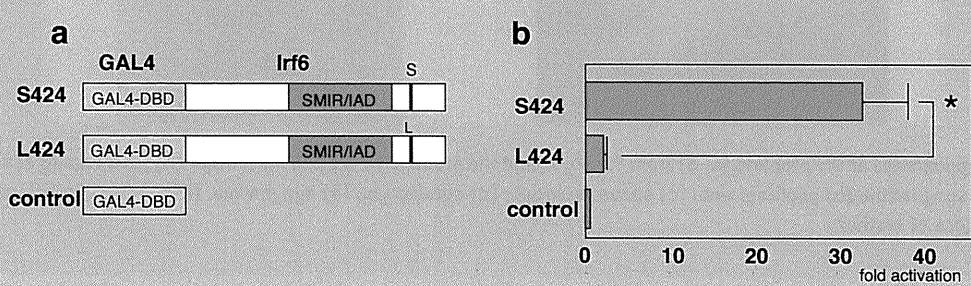

FIG. 3. Luciferase assay for transcriptional activity of mutated IRF6 protein by transient transfection of S424 into 293T cells. a: Illustration of mouse Irf6 protein (aa114–467) fused in frame to GAL4-DBD. The position of the residue 424 is indicated by S or L. GAL4-DBD, the GAL4 DNA binding domain; SMIR/IAD, SMAD/IRF or the interferon association domain. b: Luciferase value (fold activation) for each construct was measured as its ratio to a value measured for vector alone, after normalization to the co-tansfected βgal activity. The values represent the average of five experiments with standard deviation (error bars). *$P < 0.05$.

et al., 2002]. As R84 was assumed to be a critical site for DNA binding [Kondo et al., 2002], it is likely that R84L observed in Family 1 causes a change of DNA binding ability of *IRF6*, and the site R84 in the IRF6 protein is a mutational hot spot.

In Family 2, we identified another heterozygous base substitution, c.1271C>T, in the last exon of *IRF6*, and this mutation results in S424L at the C-terminus region of IRF6 protein. The S424L is a rare missense mutation found within the C-terminus domain, where only one mutation (D430N) in PPS [Kondo et al., 2002] and two mutations (P396S and R400W) in VWS [Kayano et al., 2003; Wang et al., 2003] have been reported. These findings suggest that the C-terminus region of IRF6 could have an important function, and the S424L identified in Family 2 could be associated with that function. A luciferase assay showed that the S424L mutation significantly reduces the transcriptional activity of IRF6, and two possible functions of the C-terminus are suggested. The region may act as an important domain for phosphorylation of *IRF6* via a kinase to allow correct localization as occurs in other IRFs. It has been suggested that S424 is a potential target residue of a kinase, although details are unknown [Bailey et al., 2005]. Studies on IRF3 and IRF7 have shown that phosphorylation at the C-terminus is required to unfold and dimerize them, and that the dimer is then translocated into the nucleus, where it binds with their targets and activates gene expression [Mamane et al., 1999; Sharma et al., 2003]. Alternatively, the C-terminus region could play a role in protein interaction or its regulation. This possibility is supported by a study showing an interaction of IRF6 to the mammary serine protease inhibitor (maspin) [Bailey et al., 2005]. We favor this second possibility, because a relationship between the phosphorylation state of IRF6 and subcellular localization was not supported either by immunofluorescent staining [Bailey et al., 2008] or by luciferase assay using GAL4-DBD [Little et al., 2009], and the present luciferase assay using GAL4-DBD suggests that the S424L mutation abolished transcriptional activation of mutant IRF6. However, why different mutations surrounding the SMIR domain result in either the PPS or the VWS phenotype still remains to be investigated.

The molecular mechanisms resulting in PPS and VWS and the genotype–phenotype correlations between them remain unclear. A possible explanation why mutations in the same gene cause two different syndromes is the nature of the mutations. One idea is that haploinsufficiency of *IRF6* causes VWS, while PPS develops by a dominant negative mechanism or by a situation of less than half insufficiency [Kondo et al., 2002]. According to this hypothesis, mutated IRF6 at R84 found in PPS would completely abolish the transcriptional activator function without any disturbance of localization or dimerization regulation. The R84 mutations found in Family 1 and in seven unrelated PPS families [Kondo et al., 2002] support this hypothesis. Likewise, mutations in the C-terminus region resulting in PPS, such as S424L seen in Family 2, would abolish completely the transcriptional activity. A quantitative assay reflecting intrinsic *IRF6* transcriptional activity should provide an answer. Alternatively, as the two disorders cannot always be differentiated clearly [Khan et al., 1986], they may fall into a wide clinical spectrum, resulting in little genotype–phenotype correlation.

Variable expressivity within a family is also evident, as seen in Family 1, where only the proband had typical clinical signs for PPS while other affected members had only cleft palate with or without cleft lip. Clinical severity is very different between typical PPS and VWS patients. Although these findings can be explained by an assumption that the two conditions represent each end of the spectrum, it is also plausible that various mutated IRF6 proteins would show consecutive intrinsic transcriptional activity under an influence of a modifier gene(s) and the PPS/VWS phenotypes would be distinctively categorized through thresholds according to the level of activity. All five mutations (R9W, R45Q, R84C, E349V, and P396S) reported in Japanese VWS patients [Kayano et al., 2003; Matsuzawa et al., 2004] are located at either the DNA binding domain or the SMIR domain, and there is no evidence for the existence of a Japanese-specific mutation spectrum. However, as we previously found R84C to be quite common [Matsuzawa et al., 2004], some genetic factors modifying IRF6 function might exist more frequently in the Japanese.

In conclusion, two mutations associated with PPS have been identified, c.251G>T (R84L) and c.1271C>T (S424L), one each in the two families studied. The nature of the mutations suggests R84 and S424 are biologically important sites for DNA binding ability and for transcriptional activation of IRF6, respectively. To our knowledge, this is the first report of mutations observed in Japanese PPS patients.

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The possibility of microarray-based analysis using cell-free placental mRNA in maternal plasma

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Objective The purpose of this study is to investigate a possibility of overall assessment of cell-free (CF) placental mRNAs in maternal plasma.

Methods First, placenta-predominantly expressed transcripts were selected by the analysis of GeneChip using three sets of placental tissues and corresponding maternal blood cells. Subsequently, a custom cDNA array panel of placenta-predominantly expressed transcripts was designed and used to compare the RNA profiles of maternal plasma collected from 12 preeclamptic and 12 uncomplicated pregnancies. Scatter plots for the signal intensities of the comparative cDNA hybridization revealed either unchanged or aberrant patterns.

Results We selected top 50 placenta-predominantly expressed transcripts that were >2500 times higher in placental tissues than in corresponding whole blood samples. A custom cDNA array analysis detected the aberrant pattern in five preeclamptic women with severe hypertension but not in seven preeclamptic women with mild hypertension ($P < 0.05$, Fisher's direct method). The aberrant pattern of above RNA transcripts in maternal plasma was validated by quantitative real-time reverse transcription-polymerase chain reaction. The mean (range) value of coefficient of variations in this custom array quantification was 9.4% (3.0–16.2%).

Conclusion Our custom cDNA array is expected to be useful for overall assessment of CF placental mRNAs in maternal plasma in a single experiment. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS: microarray-based analysis; cell-free placental mRNA; maternal plasma; placental status; preeclampsia; noninvasive diagnosis

INTRODUCTION

Assessment of the fetal/placental status during pregnancy is currently carried out by fetal cardiotocography, ultrasonography and/or tests using biological marker molecules, but they often produce false-positive results (Bobby, 2003). To obtain direct information regarding the fetal/placental status, conventional prenatal diagnostic procedures such as amniocentesis, cordocentesis or fetal scalp blood sampling may remain useful. However, these invasive procedures always involve the risk of serious complications, for example fetal loss, rupture of the membrane and infections. Therefore, noninvasive detection of fetus or placenta-derived molecular markers in pregnant women is desirable for accurate monitoring of the fetal/placental status. The recent discovery of cell-free (CF) placental mRNA in maternal plasma has provided possibilities for exploring placental dysfunction (Lo and Chiu, 2007; Maron and Bianchi, 2007). CF placental transcripts can be detected in maternal plasma by week 4 of gestation and have a median half-life of 14 min (Chiu *et al.*, 2006). The pregnancy specificity of CF placental mRNA has been demonstrated by its rapid clearance from the maternal plasma after delivery. Therefore, its detection in the maternal circulation appears to be a promising approach for the development of sex- and polymorphism-independent fetal/placental molecular markers for prenatal gene expression profiling. CF placental mRNA in maternal plasma is measurable by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) (Miura *et al.*, 2008), and placental transcripts are more readily detectable in plasma than in whole blood (Heung *et al.*, 2009).

The placental functions during pregnancy are regulated by a large variety of genes. Preeclampsia is a serious complication of pregnancy, and its pathogenesis is known to be associated with multiple factors, such as abnormal placentation, reduced placental perfusion, endothelial cell dysfunction and systemic vasospasm. The plasma concentrations of circulating CF placental mRNA including transcripts for corticotrophin-releasing hormone, placenta-specific gene 1 and selectin P were reported to be higher for pregnant women with preeclampsia than for normal pregnant women (Lo and Chiu, 2007; Maron and Bianchi, 2007; Purwosunu *et al.*, 2007). These findings suggest that certain different kinds of CF placental mRNA in maternal plasma can be used as markers for preeclampsia. However, overall assessment of CF placental mRNA by a single quantitative

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real-time RT-PCR analysis is difficult because of the limitation of the blood sampling volume. To resolve this technical issue, the development of a high-throughput microarray-based approach is promising. In addition, microarray comparative genomic hybridization analysis of CF fetal DNA in amniotic fluid already provides rapid screening for chromosome abnormalities with copy-number changes (Larrabee *et al.*, 2004; Miura *et al.*, 2006), though amniotic fluid cannot be obtained noninvasively. The application of a microarray-based method using CF placental mRNA in maternal plasma is feasible and may allow a noninvasive overall assessment of the placental status in a single experiment.

In this study, we initially identified placenta-predominantly expressed transcripts in maternal blood by comparisons between their expression levels in the placenta and maternal blood (Tsui *et al.*, 2004). Subsequently, we generated a custom cDNA microarray panel composed of the cDNAs of these genes. Finally, we adopted a custom array-based comparative cDNA hybridization using CF mRNA in maternal plasma to establish an overall assessment of CF placental mRNA levels in pregnant women.

MATERIALS AND METHODS

Subjects and sample collection

The study subjects comprised 12 pregnant women with preeclampsia and 12 normal pregnant women whose gestational weeks were matched with those of the preeclamptic women. CF plasma samples (12 mL) were prepared from maternal blood by a double centrifugation method. All these women attended the Department of Obstetrics and Gynecology at the Nagasaki University Hospital. Preeclampsia was defined as gestational hypertension (systolic pressure $\geq$140 mm Hg and/or diastolic blood pressure $\geq$90 mm Hg on at least two occasions during measurements after 20 weeks of gestation) with proteinuria ($\geq$0.3 g/day). Severe preeclampsia was defined by the presence of one or more of the following findings: severe gestational hypertension (systolic pressure $\geq$160 mm Hg and/or diastolic blood pressure $\geq$110 mm Hg on two occasions at least 6 h apart while the patient was on bed rest); severe proteinuria ($\geq$5 g of protein in a 24-h urine specimen, or 3 or greater in two random urine samples collected at least 4 h apart). Women in whom the onset of preeclampsia was before 32 weeks of gestation were defined as having the early-onset type, while those with onset after 32 weeks of gestation were defined as having the late-onset type.

Placental tissue samples were obtained from three normal pregnant women immediately after termination of pregnancy during the first (7–12 weeks of pregnancy), second (18–21 weeks) or third (37–39 weeks) trimesters (Figure 1), immediately placed in RNAlater™ (Ambion, Austin, TX) and stored at −80 °C until RNA extraction. Blood samples (6 mL) were collected from the women into PAXgene™ blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) before the termination of pregnancy at each trimester. All the samples

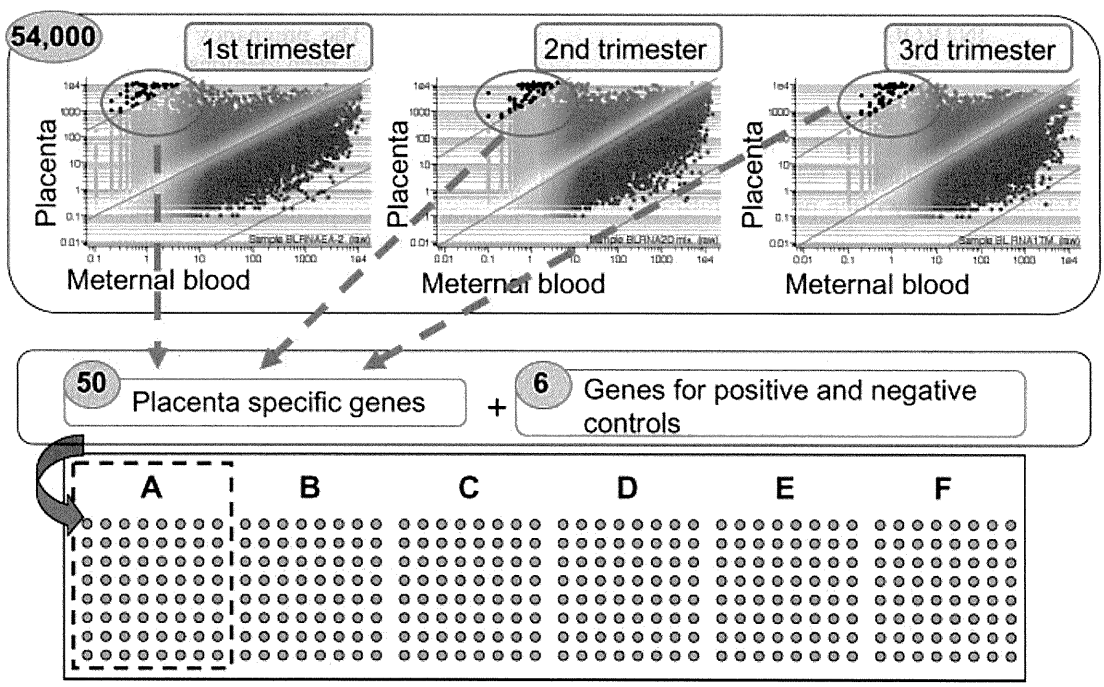

Figure 1—Outline of the strategy used for the development of a custom microarray panel of placenta-specific genes in maternal blood. Upper panel: gene expression profiles of three paired samples of placenta tissues and corresponding whole maternal blood (at the first, second and third trimesters). Black dots indicate placenta-specific transcripts exhibiting signal intensities that are >2500 times higher in the placenta in every trimester compared with the corresponding whole maternal blood samples. Middle panel: collection of 50 placenta-specific genes plus 6 positive and negative control genes. Lower panel: a custom cDNA microarray panel where 6 blocks containing a total of 56 gene-specific sequences were robotically spotted onto a single slide. The 50 placenta-specific genes were spotted in the order of their high expression levels in the placenta

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were obtained after receiving written informed consent, and the study protocol was approved by the Institutional Review Board for Ethical, Legal and Social Issues at the Nagasaki University.

Identification of placenta-predominantly expressed transcripts in the maternal circulation

Total RNA from the placental tissue samples and the peripheral blood were extracted as described, respectively, by Tsui *et al.* (2004) and Okazaki *et al.* (2007). The same amount of RNA from the placental or corresponding maternal blood sample was subjected to GeneChip® analysis (Affymetrix, Santa Clara, CA). Because the expression pattern of placental transcripts is changing during pregnancy, three sets (placental tissue and corresponding maternal blood sample) at each trimester were analyzed. For each sample, the extracted RNA (1 μg) was labeled by the one-cycle target labeling method, and biotinylated cDNA (15 μg) was hybridized to a GeneChip® Human Genome U133 Plus 2.0 (Affymetrix) according to the manufacturer's instructions. After the hybridization, the arrays were washed and stained in a GeneChip Fluidics Station 450 (Affymetrix). The chips were scanned with a GeneChip Scanner 3000 (Affymetrix) and analyzed using GeneChip Microarray Suite 5.0 (Affymetrix). The raw intensity data were analyzed with the GeneChip Operating Software (Affymetrix), and data mining was performed with the GeneSpring software (Agilent Technologies, Palo Alto, CA). The corrected signal intensities for the placental tissues and blood samples were expressed as scatter plots (Figure 1). Three sets of placental tissue and maternal blood cell samples across the three trimesters were used to look for the placenta-predominantly expressed genes that were detected in one or more out of the three profiles. The top 50 placenta-predominantly expressed transcripts exhibiting raw signal intensities that were >2500 times higher in the placental tissues than in the corresponding whole blood samples were selected according to a previously reported strategy for systematic identification of pregnancy-specific placental mRNA markers expressed in the maternal circulation (Tsui *et al.*, 2004).

Development of a custom microarray panel of placenta-predominantly expressed genes

Preparation of probe cDNAs

The 50 placenta-predominantly expressed transcripts (Nos. 1–50) and six control transcripts (Nos. 51–56) were selected as probe cDNAs to be spotted onto a custom microarray panel (Table 1, Figure 1). The control transcripts included five placenta-nonspecific genes as positive controls (Nos. 51–55) and the plasmid sequence of the pBluescriptII SK(−) vector as a negative control (No. 56). The 50 primer sets for the placenta-specific genes and 5 primer sets for the placenta-nonspecific genes (TaqMan® Gene Expression Assays) were purchased from Applied Biosystems (Foster City, CA, USA) (each assay ID is listed in Table 1). 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 (Ng *et al.*, 2003b; Okahara *et al.*, 2004). Each placental cDNA aliquot (2 μL) was subjected to 30 cycles of PCR in a total volume of 20 μL. The PCR product was cloned into the TOPO II vector (Invitrogen (Carlsbad, CA, USA)), and the plasmid DNA was extracted. Several clones for each transcript were sequenced using an ABI3100 to confirm the sequence integrity, and a total of 55 clones, 1 for each from the placenta-specific and nonspecific transcripts, were selected.

As probe DNA resources for the microarray panel, the sequences integrated into the TOPO II vector were amplified with primers that were modified to have sequences for an amino residue ($-NH_2$) at their 5′ ends. The primer sequences were as follows: topo-forward: 5′-agtgtgctggaattcgccct-3′; topo-reverse: 5′-gatatctgcagaattcgccct-3′. After purification using a Microcon YM-10/30/100 (Millipore Corporation, Billerica, MA), the concentration of the collected DNA was 138–580 ng/μL and each volume was 50–100 μL. The PCR amplifications involved 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s in a 100-μL mixture containing 1 ng of cDNA, 10 pM of primers (topo-forward and topo-reverse), 250 μM dNTP, 0.5 U of Ex Taq polymerase (TaKaRa Bio Inc., Tokyo, Japan) and 10× PCR buffer (TaKaRa Bio Inc.). PCR amplification was performed four times for each cDNA clone.

DNA spotting

The PCR products were dissolved in distilled water, and an equal volume of spotting solution DSP0050 (Matsunami, Osaka, Japan) was added. The final concentration of the PCR products was 0.15 μg/μL. The resulting DNA samples (0.15 μg/μL at 200 pL/spot) were robotically spotted using an inkjet printing technique (NGK, Nagoya, Japan) in six blocks onto CodeLink™ activated slides (Amersham Biosciences, Piscataway, NJ). The design of the custom cDNA microarray is shown in Figure 1.

Microarray-based comparative cDNA hybridization between samples from preeclamptic women and control pregnant women

cDNA preparation from the women and T7-based CF mRNA amplification

CF mRNA was extracted as described previously (Zhong *et al.*, 2008) and amplified by a T7-based RNA amplification method (Abe *et al.*, 2003), because the concentration of CF mRNA in maternal plasma was too low for direct use in a microarray analysis (Figure 2). A total of 56 specific primers [RNA amplification primers (RAPs)]

Table 1—Fifty genes highly expressed in the placenta compared with the maternal blood

	Gene symbol (name)	GenBank accession number	Assay ID	Chromosome localization	Gene expression[a]	Gene function	CV value[b] (custom array) (%)	CV value (real-time RT-PCR) (%)
1	LUM (lumican)	NM_002345.3	Hs00158940_m1	12q21.3-q22	P	A member of the small leucine-rich proteoglycan family	12.0	1.7
2	RAI14 (retinoic acid induced 14)	NM_015577.1	Hs00210238_m1	5p13.3-p13.2	P	Retinoic acid regulated gene	14.9	3.3
3	CDH1 [cadherin 1, type 1, E-cadherin (epithelial)]	NM_004360.2	Hs00170423_m1	16q22.1	P	Calcium ion-dependent cell adhesion molecule	7.8	5.8
4	CSRP2 (cysteine and glycine-rich protein 2)	NM_001321.1	Hs00426717_m1	12q21.1	P	Member of the CRP (cysteine- and glycine-rich protein) family of LIM (Lin/Isl/Mec) domain proteins	13.2	4.8
5	ERVWE1 [endogenous retroviral family W, env(C7), member 1, syncytin 1]	NM_014590.3	Hs00205893_m1	7q21-q22	P	Part of an HERV provirus, which is expressed in the placental syncytiotrophoblast and is involved in fusion of the cytotrophoblast cells to form the syncytial layer of the placenta	10.2	3.1
6	INHBA (inhibin, beta A)	NM_002192.2	Hs00170103_m1	7p15-p13	P	Growth/differentiation hormone	17.2	4.7
7	PSG5 (pregnancy-specific beta-1-glycoprotein 5)	NM_002781.2	Hs00818332_m1	19q13.2	P	A group of molecules that are mainly produced by the placental syncytiotrophoblasts during pregnancy	12.1	10.6
8	TFPI (tissue factor pathway inhibitor)	NM_001032281.2	Hs00196731_m1	2q31-q32.1	P	A protease inhibitor that regulates the tissue factor-dependent pathway of blood coagulation	6.9	6.0
9	INSL4 (insulin-like 4)	NM_002195.1	Hs00171411_m1	9q24	P	A member of the insulin superfamily	9.1	4.6
10	LEP (leptin)	NM_000230.1	Hs00174877_m1	7q31.3	P	Secreted by adipocytes and by placenta	13.0	9.1
11	TFPI2 (tissue factor pathway inhibitor 2)	NM_006528.2	Hs00197918_m1	7q22	P	A placental glycoprotein that inhibits plasmin, trypsin and thrombin	7.3	8.9
12	GH1 (growth hormone 1, transcript variant 1)	NM_000515.3	Hs00236859_m1	17q24.2	P	A member of the somatotrophin/prolactin family of hormones which play an important role in growth control	13.1	2.6
13	ADAM12 (a disintegrin and metalloproteinase domain 12)	NM_003474	Hs01106104_m1	10q26.3	P	Candidate regulator of trophoblast fusion	12.7	5.2
14	ANGPT2 (angiopoietin 2)	NM_001147.1	Hs00169867_m1	8p23.1	P	An antagonist of angiopoietin 1 (ANGPT1) and endothelial TEK (endothelial-specific receptor tyrosine kinase) tyrosine kinase (TIE-2 (tunica internal endothelial cell kinase 2), TEK)	10.9	6.1
15	GH1 (growth hormone 1), transcript variant 4	NM_022561.2	Hs00737955_m1	17q24.2	P	A member of the somatotrophin/prolactin family of hormones that play an important role in growth control	11.4	7.6
16	PSG9 (pregnancy-specific beta-1-glycoprotein 9)	NM_002784.2	Hs00358192_m1	19q13.2	P	A group of molecules that are mainly produced by the placental syncytiotrophoblasts during pregnancy	16.2	7.7
17	CGA (glycoprotein hormones, alpha polypeptide)	NM_000735.2	Hs00174938_m1	6q12-q21	P	The alpha subunit and belongs to the glycoprotein hormones alpha chain family	12.0	5.2
18	KISS1 (KiSS-1 metastasis suppressor)	NM_002256.2	Hs00158486_m1	1q32	P	A putative role in the regulation of events downstream of cell-matrix adhesion	8.9	2.8

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Table 1—(*Continued*)

	Gene symbol (name)	GenBank accession number	Assay ID	Chromosome localization	Gene expression[a]	Gene function	CV value[b] (custom array) (%)	CV value (real-time RT-PCR) (%)
19	CAPN6 (calpain 6)	NM_014289.2	Hs00560073_m1	Xq23	P	Ubiquitous, well-conserved family of calcium-dependent, cysteine proteases	12.5	6.1
20	PSG6 (pregnancy-specific beta-1-glycoprotein 6)	NM_001031850.1	Hs00747417_m1	19q13.2	P	A group of molecules that are mainly produced by the placental syncytiotrophoblasts during pregnancy	9.9	6.3
21	TIMP3 (TIMP metallopeptidase inhibitor 3)	NM_000362.4	Hs00165949_m1	22q12.1-q13.2	P	Inhibitors of the matrix metalloproteinases	5.8	3.3
22	FBLN1 (fibulin 1)	NM_001996.2	Hs00242546_m1	22q13.3	P	A secreted glycoprotein that becomes incorporated into a fibrillar extracellular matrix	13.6	9.4
23	PRG2 (plasticity-related gene 2)	NM_002728.4	Hs00794928_m1	19p13.3	P	Lipid phosphate phosphatase family	8.4	3.0
24	CYP19A1 (cytochrome P450, family 19, subfamily A, polypeptide 1)	NM_031226.1	Hs00240671_m1	15q21.1	P	A member of the cytochrome P450 superfamily of enzymes	8.4	4.9
25	PSG3 (pregnancy-specific beta-1-glycoprotein 3)	NM_021016.3	Hs00360732_m1	19q13.2	P	A group of molecules that are mainly produced by the placental syncytiotrophoblasts during pregnancy	6.0	2.8
26	PPAP2B (phosphatidic acid phosphatase type 2B)	NM_177414.1	Hs00170359_m1	1pter-p22.1	P	A member of the phosphatidic acid phosphatase family	12.6	0.6
27	P11 (26 serine protease, placental protein 11)	NM_006025.2	Hs00195731_m1	12q13.1	P	A serine protease specifically expressed in the syncytiotrophoblast	6.9	4.5
28	PAGE4 (P antigen family, member 4)	NM_007003.2	Hs00199655_m1	Xp11.23	P	Expressed in a variety of tumors and in some fetal and reproductive tissues including placenta, a member of the GAGE (G antigen) family	9.6	9.3
29	SMARCA1 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1)	NM_003069.2	Hs00161922_m1	Xq25	P	A member of the SWI/SNF (switch/sucrose nonfermentation) family of proteins	4.7	4.9
30	COL1A2 (collagen, type I, alpha 2)	NM_000089.3	Hs00164099_m1	7q22.1	P	The pro-alpha 2 chain of type I collagen whose triple helix comprises two alpha 1 chains and one alpha 2 chain	8.6	0.9
31	GULP1 (GULP, engulfment adaptor PTB (phosphotyrosine binding) domain containing 1)	NM_016315.2	Hs00169604_m1	2q32.3-q33	P	An evolutionarily conserved adaptor protein required for efficient engulfment of apoptotic cells by phagocytes	8.4	6.2
32	PLEKHC1 (pleckstrin homology domain containing, family C member 1)	NM_006832.1	Hs00235033_m1	14q22.1	P	A component of ECM (cell-extracellular matrix) structures in mammalian cells	6.9	1.8

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Table 1—(*Continued*)

	Gene symbol (name)	GenBank accession number	Assay ID	Chromosome localization	Gene expression[a]	Gene function	CV value[b] (custom array) (%)	CV value (real-time RT-PCR) (%)
33	PKIB [protein kinase (cAMP-dependent, catalytic) inhibitor beta]	NM_032471.4	Hs00261162_m1	6q22.31	P	A member of the cAMP-dependent protein kinase inhibitor family	6.6	4.8
34	CXCL14 (chemokine (C-X-C motif) ligand 14)	NM_004887.3	Hs00171135_m1	5q31	P	The cytokine gene family which encode secreted proteins involved in immunoregulatory and inflammatory processes	6.9	4.6
35	PEG3 (paternally expressed 3)	NM_006210.1	Hs00377844_m1	19q13.4	P	A Kruppel-type ZNF (zinc finger protein) protein, high levels of PEG3 in the human placenta and localized the signal to the layer of villous cytotrophoblast cells	5.6	3.7
36	ESRRG (estrogen-related receptor gamma)	NM_206594.1	Hs00155006_m1	1q41	P	A member of the steroid/thyroid/retinoid receptor superfamily	10.8	5.9
37	EBI3 (Epstein-Barr virus induced 3)	NM_005755.2	Hs00194957_m1	19p13.3	P	A secreted glycoprotein belonging to the hematopoietin receptor family	7.0	3.3
38	HSD3B1 (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1)	NM_000862.2	Hs00426435_m1	1p13.1	P	Type I enzyme that is expressed mainly in the placenta and peripheral tissues	6.3	4.7
39	PAPPA (pregnancy-associated plasma protein A)	NM_002581.3	Hs00361692_m1	9q33.2	P	A large zinc glycoprotein of placental origin	10.4	6.7
40	PLAP (alkaline phosphatase, placental)	NM_001632.3	Hs01654626_s1	2q37	P	A membrane-bound glycosylated enzyme, which appears in the serum during pregnancy	9.4	6.1
41	CSH1 (chorionic somatomammotropin hormone 1 (placental lactogen))	NM_022640.2	Custom primer[c]	17q24.2	P	A member of the somatotrophin/prolactin family of hormones and plays an important role in growth control	3.5	1.0
42	SLC7A2 (solute carrier family 7 (cationic amino acid transporter, y+ system), member 2)	NM_003046.3	Hs00161809_m1	8p22-p21.3	P	Member of the APC (amino acid-polyamine-organocation) family of transporters	9.3	5.6
43	EFEMP1 (EGF-containing fibulin-like extracellular matrix protein 1)	NM_001039348.1	Hs00244575_m1	2p16	P	Extracellular matrix protein, which is expressed in many tissues but it is not expressed in brain and lymphocytes	11.1	3.7
44	CGB (chorionic gonadotrophin, beta polypeptide)	NM_000737.2	Hs00361224_gH	19q13.32	P	A member of the glycoprotein hormone beta chain family	10.5	10.4
45	COL3A1 (collagen, type III, alpha 1)	NM_000090.3	Hs00164103_m1	2q31	P	The pro-alpha 1 chains of type III collagen	13.0	6.5
46	LIFR (leukemia inhibitory factor receptor alpha)	NM_002310.3	Hs01123581_m1	5p13-p12	P	A protein that belongs to the type I cytokine receptor family	6.9	8.0

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Table 1—(*Continued*)

	Gene symbol (name)	GenBank accession number	Assay ID	Chromosome localization	Gene expression[a]	Gene function	CV value[b] (custom array) (%)	CV value (real-time RT-PCR) (%)
47	SERPINE1 [serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1, plasminogen activator inhibitor-1 (PAI1)]	NM_000602.1	Hs00167155_m1	7q21.3-q22	P	This inhibitor acts as 'bait' for tissue plasminogen activator, urokinase and protein C	4.4	5.3
48	HERV-FRD (human endogenous retrovirus FRD envelope protein, syncytin 2)	NM_207582.1	Hs01652148_m1	6p24.1	P	HERV-FRD envelop protein, which is expressed predominantly in placenta, has a potential role in placenta formation	5.7	3.8
49	PSG2 (pregnancy-specific beta-1-glycoprotein 2)	NM_031246.1	Hs01652779_m1	19q13.1-q13.2	P	A group of molecules that are mainly produced by the placental syncytiotrophoblasts during pregnancy	8.5	10.4
50	HERV-FRD (human endogenous retrovirus FRD envelope protein, syncytin 2)	NM_207582.1	Custom primer[c]	6p24.1	P	HERV-FRD env protein, which is expressed predominantly in placenta, has a potential role in placenta formation	3.0	1.9
51	GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	NM_002046.3	Hs99999905_m1	12p13	P&B	A kinase involved in the glycolysis-dependent endogenous phosphorylation of the alpha 1 subunit of the GABA-A receptor	7.6	8.4
52	MRPS16 (mitochondrial ribosomal protein S16)	NM_016065.3	Hs00831691_s1	10q22.1	P&B	Protein synthesis within the mitochondrion	12.6	10.0
53	EEF1A2 (eukaryotic translation elongation factor 1 alpha 2)	NM_001402.5	Hs00265885_g1	20q13.3	P&B	Isoform of the alpha subunit of the elongation factor 1 complex	8.7	1.5
54	IL8RA (interleukin 8 receptor, alpha)	NM_000634.2	Hs00174146_m1	2q35	P&B	A member of the G protein-coupled receptor family	13.8	9.4
55	P2RY13 (purinergic receptor P2Y, G protein coupled, 13)	NM_023914.2	Hs03043902_s1	3q24	P&B	The family of G protein-coupled receptors	7.5	5.1
56	Blue script plasmid DNA	—	—	—	—	No DNA sequence in human	—	—

[a] P indicates dominantly expressed in placental tissues and P&B indicates transcripts equally expressed in both placenta and blood.
[b] Coefficient of variations that is the ratio of the standard deviation to the mean value.
[c] TaqMan probe and gene-specific primers were described as previously (Ng *et al.*, 2003b; Okahara *et al.*, 2004).

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were designed. These RAPs had a 45-bp T7 RNA polymerase consensus sequence at their 5′ end and also contained approximately 20-bp sequences of the genes corresponding to the cDNAs in the spots (their sequences are available on request). An antisense sequence was also designed for each spotted probe containing about 50 bp at its 3′ end. RNA amplification was performed as follows. CF mRNA was reverse transcribed using the RAPs and converted into double-stranded cDNA. To obtain a large amount of each target RNA, the double-stranded cDNA was used as a template for transcription with T7 RNA polymerase, and the amplified RNA was dissolved in 30 μL of diethylpyrocarbonate-treated (DEPC) H_2O. The specificity of the cDNA amplification in maternal plasma was confirmed by sequencing analysis.

Labeling and hybridization of CF placental mRNA

Comparative cDNA hybridization of CF placental mRNA from the preeclamptic women and normal control women was performed using the cDNA microarray panel (Figure 2). Amplified test cDNA (2 μg) from preeclamptic women and reference cDNA from normal control women were labeled with the fluorescent dyes Cy-5 and Cy-3, respectively. The two types of labeled cDNAs (final volume, 40 μL) were hybridized competitively with the custom cDNA microarray panel in a hybridization buffer composed of 5× saline-sodium citrate (SSC) and 0.5% sodium dodecyl sulfate (SDS) at 42 °C for 16 h. After the comparative hybridization, each panel was washed three times with buffer solutions composed of 2× SSC and 0.1% SDS, 2× SSC, 1× SSC and 0.5× SSC, respectively. The panels were scanned with a G2565AA Microarray Scanner System (Agilent Technologies) and analyzed using GenePix Pro Ver. 4.0.1.17 (Axon Instruments, Foster City, CA). The data for the comparative cDNA hybridization were corrected by the difference between the signal intensities of the test and reference cDNAs. The mean value of the signal intensities for each spot was calculated from the data of the six blocks on each slide (Figure 1). The corrected signal intensities for preeclamptsia and control pregnancies were expressed as scatter plots. Plasma samples from the only control pregnancies were used for the computation of the coefficient of variations (CV) values, which is the ratio of the standard deviation to the mean value, in this custom array quantification.

Quantitative real-time RT-PCR

One-step quantitative real-time RT-PCR assay was performed using ABI 7900T Sequence Detector (Perkin-Elmer, Foster City, CA) as described previously (Ng *et al.*, 2003b). The PCR products of 56 genes were cloned into the TOPO II vector (Invitrogen), respectively. Each of the extracted plasmid DNA was used

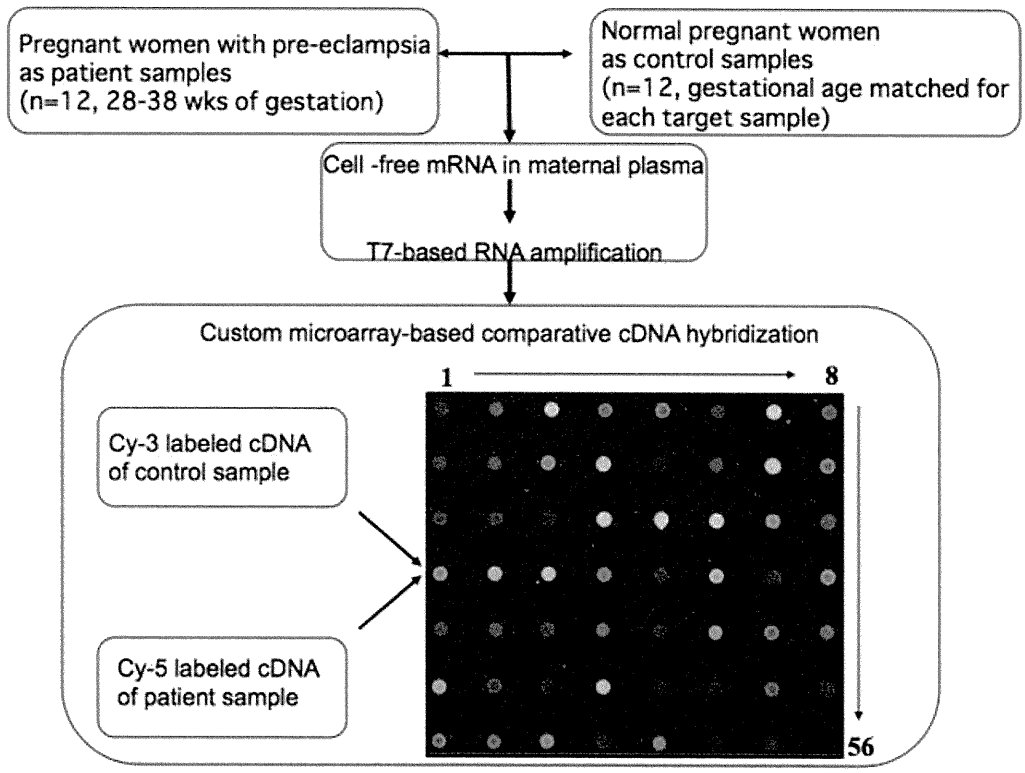

Figure 2—Outline of the custom microarray-based comparative cDNA hybridization. CF mRNA was extracted from 1.2 mL of individual plasma samples from 12 pregnant women with preeclampsia (27–38 weeks of gestation) and 12 normal pregnant women with matched gestational ages. The CF mRNA amplified by a T7-based RNA amplification method was subjected to comparative cDNA hybridization, in which amplified cDNA (2 μg) from preeclamptic women and control amplified cDNA from normal pregnant women were labeled with the fluorescent dyes Cy-5 and Cy-3, respectively. The same volumes of the labeled products were comparatively hybridized with the custom cDNA microarray panel. The mean values of six signal intensities for each sample were expressed as a scatter plot

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for a calibration curve of each gene. RT-PCR validation of the 12 normal control pregnancies was done for all 56 genes.

RESULTS

Identification of placenta-predominantly expressed genes in maternal plasma by microarray analysis, and development of a microarray panel from their cDNAs

We identified 50 placenta-specific transcripts that showed >2500 times higher expression in the placental tissues than in the corresponding whole blood samples in the respective trimesters of pregnancy (Table 1). Using the cDNAs of these transcripts, we created a custom cDNA array panel (Figure 1). To confirm whether a single experiment using the panel led to an accurate result, equal volumes of the same placental cDNA sample labeled with either Cy-5 or Cy-3 were hybridized in a comparative manner. Approximately equal signal intensities from Cy-5 and Cy-3 for the placental cDNA were successfully detected on all 55 spots, comprising the 50 placental and 5 positive control transcripts (*GAPDH, MRPS16, EEF1A2, IL8RA* and *P2RY13*), while no signal was obtained for the 56th spot as a negative control (the plasmid sequence from pBluescriptII SK(−) vector) (Figure 2).

The accuracy of the total plasma CF mRNA amplification using the T7-based RNA amplification method was confirmed by quantitative real-time PCR measurements (Abe *et al.*, 2003). Specifically, the average cycle threshold (Ct) values of the nonamplified positive control transcripts CF *GAPDH* mRNA, CF *MRPS16* mRNA, CF *EEF1A2* mRNA, CF *IL8RA* mRNA and CF *P2RY13* mRNA were 32.292, 32.211, 31.981, 33.017 and 32.143, while the Ct values for the same mRNAs after amplification were 24.927, 24.833, 23.163, 25.933 and 24.812, respectively. Similar results between amplified and nonamplified CF mRNA were also confirmed on a panel of placental RNA transcripts in maternal plasma. These data indicated that the total CF mRNA was reproducibly amplified using the T7-based RNA amplification method, which generated gene expression profiles that were comparable with the nonamplified total RNA as previously reported (Abe *et al.*, 2003). Valid signals for the amplified CF mRNA samples on the 55 spots were also detected, but not for the 56th spot comprising the negative control.

Identification of 17 different transcripts with increased or decreased levels of maternal plasma CF mRNA in severely hypertensive preeclamptic women

We created scatter plots for the signal intensities of the comparative hybridization between the target cDNA from the CF mRNA of preeclamptic women and the same amount of control cDNA from the CF mRNA of control pregnant women. The plots showed either an unchanged or an aberrant pattern (Figure 3). The aberrant pattern was defined when the signal intensity of the target cDNA was increased or decreased by twofold or more compared with the intensity of the gestational age-matched control cDNA. The aberrant pattern was specifically observed in five preeclamptic women with severe hypertension (cases 1–5) and not in the seven preeclamptic women with mild hypertension (cases 6–12) (Table 2) ($P < 0.05$, Fisher's direct method). Therefore, the aberrant pattern was associated with preeclampsia with severe hypertension. There were no correlations between the aberrant pattern and the proteinuria severity or disease onset.

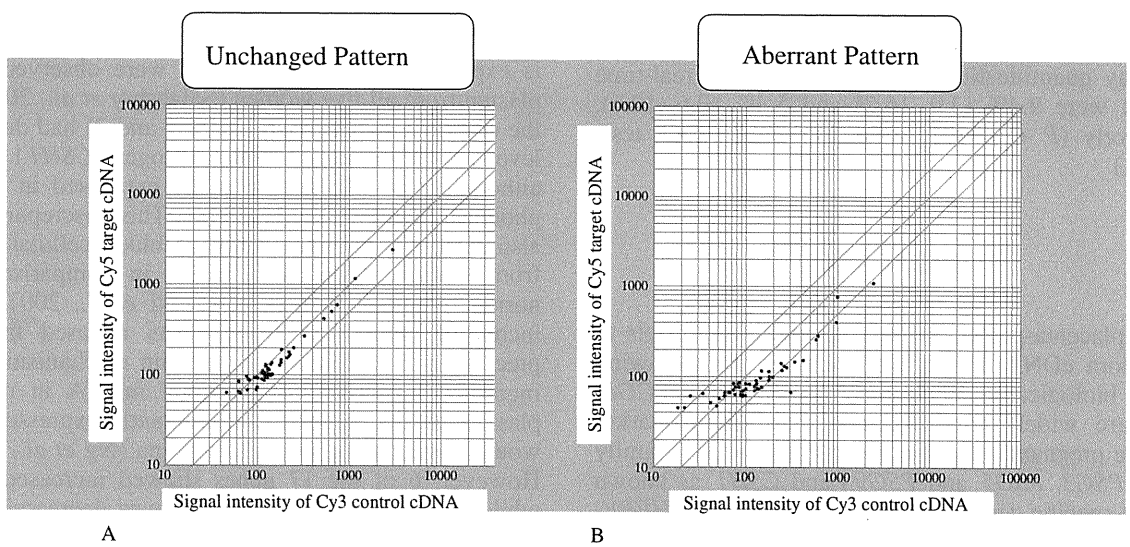

Figure 3—Scatter plots of the signal intensities obtained by comparative hybridization on the custom microarray between target cDNA from CF mRNA in plasma samples from preeclamptic women and control cDNA from normal control pregnant women. (a) Unchanged pattern showing that all the plots are within ±twofold from their equal intensity value. (c) Aberrant pattern showing that some plots are above or below the twofold line

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Table 2—Associations between the signal intensity patterns of CF placenta-specific mRNA and the severity of preeclampsia

Cases	Hypertension	Proteinuria	Disease onset	Signal-intensity patterns
1	Severe	Mild	Late	Aberrant
2	Severe	Severe	Early	Aberrant
3	Severe	Mild	Early	Aberrant
4	Severe	Mild	Late	Aberrant
5	Severe	Severe	Early	Aberrant
6	Mild	Mild	Early	Unchanged
7	Mild	Severe	Late	Unchanged
8	Mild	Mild	Early	Unchanged
9	Mild	Severe	Late	Unchanged
10	Mild	Severe	Early	Unchanged
11	Mild	Mild	Early	Unchanged
12	Mild	Severe	Late	Unchanged

The aberrant plots for the five preeclamptic women with severe hypertension included 17 different transcripts (Table 3). Among the 17 transcripts, 6 transcripts (*TFPI, ADAM12, PSG9, PSG5, CYP19A1* and *PLAP*) exhibited twofold or much higher intensities, while the remaining 11 transcripts (*CDH1, ERVWE1, INHBA, PPAP2B, P11, PKIB, CXCL14, PEG3, ESRRG, CSH1* and *HERV-FRD*) exhibited twofold or much lower intensities. However, all five severely hypertensive preeclamptic women with the aberrant pattern had different combinations of increased or decreased transcripts (Table 3). For example, cases 1 and 2 showed aberrant levels for most of the 17 placenta-specific genes, while cases 3–5 showed aberrant levels of only two or three of the genes. In particular, *TFPI* mRNA increases were detected in three of the five women, *PSG9* mRNA increases in four of the women and a *PSG5* mRNA increase in one woman. Decreased levels of *PPAP2B* mRNA were detected in four of the five women (Table 3). The levels of amplified CF mRNAs in maternal plasma were also measured by quantitative real-time RT-PCR, and the aberrant pattern of more than 17 placental transcripts in maternal plasma between preeclamptic and uncomplicated women was validated (Table 3). The mean (range) value of CV in this custom array quantification and the quantitative real-time RT-PCR were 9.4% (3.0–16.2) and 5.4% (0.6–10.6), respectively ($P < 0.0001$, Wilcoxon signed rank test) (Table 1).

DISCUSSION

The 50 placenta-predominantly expressed transcripts in our custom cDNA array panel included the following genes in biological molecules: *INHBA, PAPPA* and *CGB*, which are widely used for maternal serum marker tests; the pregnancy-specific glycoprotein (PSG) family (*PSG2, PSG3, PSG5* and *PSG9*) and *CSH1* as known placenta-specific genes; *ADAM12, ERVWE1* and *HERV-FRD* as pathological genes for trophoblast fusion and *TFPI*, a gene related to the coagulation system. The aberrant plots of our custom array analysis included 17 different transcripts (Table 3), which have specific functions as follows: roles in pregnancy (*PSG9, PSG5, PLAP, PPAP2B, PKIB, INHBA* and *CSH1*) (Zheng *et al.*, 2000; Escalante-Alcalde *et al.*, 2003; Salahshor *et al.*, 2005; Kumpel *et al.*, 2008); participation in trophoblast fusion events (*ADAM12, ERVWE* and *HERV-FRD*) (Mi *et al.*, 2000; Blaise *et al.*, 2003; Pötgens *et al.*, 2004; Gack *et al.*, 2005; Pang *et al.*, 2009); roles in the coagulation pathway (*TFPI*) and cell adhesion (*CDH1*) (Abdel Gader *et al.*, 2006); a member of the cytokine gene family (*CXCL14*) (Kurth *et al.*, 2001), aromatases (*CYP19A1* and *ESRRG*) (Shimodaira *et al.*, 2008), imprinting gene (*PEG3*) and unknown functions but highly expressed in the human placenta (*P11*) (Hiby *et al.*, 2001). Therefore, our custom cDNA array panel may be applied as a new tool for *in vivo* assessment and monitoring of placental functions, and the aberrant levels of CF mRNA in this study may reflect a placental condition of preeclampsia with severe hypertension.

The five severely hypertensive preeclamptic women had different combinations of increased or decreased levels of CF placenta-predominantly expressed mRNA (Table 3). These observations suggest that, when estimating the condition of preeclampsia using CF placental mRNAs, an analysis with multiple molecular markers is absolutely necessary. Furthermore, increased levels of CF *PSG* (including *PSG9* or *PSG5*) mRNA, which is expressed in the trophoblast, were observed in the plasma from all five women (Salahshor *et al.*, 2005). On the contrary, two women (cases 1 and 2) had decreased levels of CF human placental lactogen (*CSH1*) mRNA, although it is also known to be expressed in the trophoblast (Kumpel *et al.*, 2008). This discrepancy was also seen in a previous study on blood cellular mRNA from women with preeclampsia in comparison with normal pregnant women (Okazaki *et al.*, 2007). If an increased number of trophoblasts released from the preeclamptic placenta was the main mechanism for the increased levels of CF placental mRNA in maternal plasma, all the placenta-predominantly expressed genes would have shown such phenomena (Ng *et al.*, 2003a). However, 6 of the 17 genes showed increased levels, while the remaining 11 genes showed decreased levels. These findings suggest that the maternal plasma levels of CF mRNA are probably caused by functional alterations of placenta affected by preeclampsia. Our custom microarray-based analysis may have the potential to identify novel genes for susceptibility to preeclampsia.

Table 3—Seventeen genes showing aberrant levels in maternal plasma in cases of preeclampsia with severe hypertension

Increased or reduced level of circulating CF mRNA	Genes	Accession no.	Chromosomal localization	Mean values of relative plasma mRNA levels by custom array and RT-PCR[a]				
				Case 1	Case 2	Case 3	Case 4	Case 5
Increased	TFPI	NM_001032281.2	2q31-q32.1	2.43 (3.12)	2.52 (2.82)			2.33 (2.67)
	ADAM12	NM_003474	10q26.3	2.45 (3.11)				
	PSG9	NM_002784.2	19q13.2	4.66 (5.31)	2.26 (3.74)	3.36 (4.42)	2.45 (2.89)	
	PSG5	NM_002781.2	19q13.2					2.42 (3.28)
	CYP19A1	NM_031226.1	15q21.1			2.05 (2.65)		
	PLAP	NM_001632.3	2q37			3.92 (3.51)		
Reduced	CDH1	NM_004360.2	16q22.1	0.46 (0.31)	0.44 (0.45)			
	ERVWE1	NM_014590.3	7q21-q22	0.44 (0.36)	0.42 (0.29)			
	INHBA	NM_002192.2	7p15-p13	0.22 (0.19)	0.43 (0.34)			
	PPAP2B	NM_177414.1	1pter-p22.1	0.45 (0.37)	0.42 (0.34)		0.43 (0.37)	0.42 (0.44)
	P11	NM_006025.2	12q13.1	0.45 (0.31)	0.41 (0.42)			
	PKIB	NM_032471.4	6q22.31		0.45 (0.38)			
	CXCL14	NM_004887.3	5q31		0.46 (0.39)			
	PEG3	NM_006210.1	19q13.4	0.47 (0.41)	0.45 (0.37)			
	ESRRG	NM_206594.1	1q41		0.47 (0.36)			
	CSH1	NM_022640.2	17q24.2	0.43 (0.35)	0.48 (0.32)			
	HERV-FRD	NM_207582.1	6p24.1	0.41 (0.31)	0.42 (0.43)			

[a] The upper values showed Cy5-labeled target cDNA/Cy3-labeled control cDNA ratio by custom array, the lower values in the parenthesis the circulating plasma mRNAs levels in preeclamptic woman/those in uncomplicated woman ratio by quantitative RT-PCR.

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Among the genes showing aberrant levels of transcripts in the plasma from the five women with hypertensive preeclampsia, some may be candidates for such novel genes. Further examinations of the above 17 candidate genes for preeclampsia with severe hypertension, such as association studies on genetic variations, may provide clues toward elucidating the pathogenetic mechanism of preeclampsia.

For the clinical use of our custom cDNA array in the future, it is noteworthy that there are some limitations in this study. Crucial genes for the pathogenesis of preeclampsia may not unique to placental RNA. Our custom microarray panel does not contain genes for vascular endothelial growth factor (*VEGF*), its receptors (*VEGFR1* and *Flt-1*) or endoglin (*ENG*), for which aberrant levels of CF mRNA have been reported to be correlated with the severity of hypertension and proteinuria (Purwosunu *et al.*, 2008). Some genes, which play roles in preeclampsia and have expression in both placental tissues and blood cells, may be filtered out at the screening stage. In addition, the case number of preeclamptic women is not enough for the comparison of gene expression levels. For the clinical use of our custom array in the future as diagnostic tool, we have to add more causative genes associated with preeclampsia to our custom array, and the study on more plasma samples will be necessary to make meaningful conclusions about applications in preeclampsia. In the quantification of mRNA, the CV values in quantitative real-time RT-PCR were better than those in custom cDNA array quantification. The tool with higher diagnostic sensitivity should be used for screening to minimize false negatives. As compared with quantitative real-time RT-PCR analysis, the current version of our custom array with poorer CV would tend to be less sensitive in detecting quantitative changes and therefore would be inappropriate as a screening tool. However, overall assessment of CF placental mRNA by quantitative real-time RT-PCR is difficult because of the limitation of blood sampling volume. When the CV value of our custom array is improved to be lower than RT-PCR analysis in future, our custom cDNA array may be used to screen the profiles of plasma CF placental mRNA in a single experiment, and the results showing aberrant pattern is likely to be confirmed by RT-PCR analysis.

In summary, we first selected 50 placenta-predominantly expressed transcripts by comparisons of the GeneChip signal intensities between placental tissues and the corresponding whole blood samples. Using the cDNAs of these genes, we then designed a custom cDNA microarray panel. Finally, the custom cDNA array was used to compare the plasma RNA profiles between preeclamptic and uncomplicated pregnancies. The custom cDNA array analysis successfully identified 17 different transcripts with increased or decreased levels of maternal plasma CF mRNA specifically in preeclamptic women with severe hypertension. Our approach using a custom cDNA array may lead to establish a noninvasive method for overall assessment of CF placental mRNA in maternal plasma in a single experiment.

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Original article

Familial brain arteriovenous malformation maps to 5p13–q14, 15q11–q13 or 18p11: Linkage analysis with clipped fingernail DNA on high-density SNP array

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ABSTRACT

Familial arteriovenous malformations (AVM) in the brain is a very rare disease. It is defined as its occurrence in two or more relatives (up to third-degree relatives) in a family without any associated disorders, such as hereditary hemorrhagic telangiectasia. We encountered a Japanese family with brain AVM in which four affected members in four successive generations were observed. One DNA sample extracted from leukocytes of the proband and ten DNA samples from clipped finger nails of other members were available. A genome-wide linkage analysis was performed on this pedigree using Affymetrix GeneCip 10K 2.0 Xba Array and MERLIN software. We obtained sufficient performance of SNP genotyping in the fingernail samples with the mean SNP call rate of 92.49%, and identified 18 regions with positive LOD scores. Haplotype and linkage analyses with microsatellite markers at these regions confirmed three possible disease-responsible regions, i.e., 5p13.2–q14.1, 15q11.2–q13.1 and 18p11.32–p11.22. Sequence analysis was conducted for ten selected candidate genes at 5p13.2–q14.1, such as *MAP3K1*, *DAB2*, *OCLN*, *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EGFLAM*, *ERBB2IP*, and *PIK3R1*, but no causative genetic alteration was detected. This is the first experience of adoption of fingernail DNA to genome-wide, high-density SNP microarray analysis, showing candidate brain AVM susceptible regions.

1. Introduction

Arteriovenous malformation (AVM) in the brain is a disease defined by the presence of arteriovenous shunt(s) through a nidus of coiled and tortuous vascular connections between feeding arteries and draining veins within the brain parenchyma [10]. This vascular malformation is thought to be congenital, and develops before or after birth [7] from a residual of the primitive artery–vein connection. Its most common symptom is intracranial hemorrhage with an estimated risk of 1.3–3.9% yearly after the diagnosis of AVM [4]. Other signs may include intractable seizures, headache and ischemic steal syndrome. The prevalence of AVM is estimated to be approximately 0.01% and the detection rate ranges between 1.12 and 1.34 per 100,000 person years [7,10]. Although most cases of AVM are sporadic, a total of 53 patients from 25 families have been reported [27]. Familial brain AVM is defined when it occurs in two or more relatives (up to third-degree relative) in a family without associated disorders such as hereditary hemorrhagic telangiectasia (HHT), is autosomal dominant multisystemic vascular dysplasia [9,27]. It is plausible that familial cases are more frequent and could be overlooked because of asymptomatic conditions in other relatives.

Although several causative genes have been elucidated in some heritable syndromic AVM [2,3,5,6,12,13,17,20,21,23,26], molecular genetic studies of familial or sporadic AVM remain scant. HHT type 1 (HHT1) and HHT type 2 (HHT2) are known to be caused by mutations in *ENG* at 9q34.11 and *ACVRL1* (or *ALK1*) at 12q13.13, respectively [12,17]. Mutations in *RASA1* at 5q14.3 cause capillary malformation-arteriovenous malformation (CM-AVM) [3,6,20,21,26] characterized by small, round-to-oval, pink-red and multiple CM: one-third of CV-AVM patients also has fast-flow lesions such as AVM. Mutations in *PTEN* have been implicated in PTEN hamartoma tumor syndromes including Bannayan–Riley–Ruvalcaba syndrome, in which AVM

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occasionally presents [23]. Three genes, *KIRIT1* (*CCM1*) [13] at 7q21.2, *MGC4607* (*CCM2*) [5] at 7p13 and *PDCD10* (*CCM3*) [2] at 3q26.1, are responsible for cerebral cavernous malformation (hamartomatous vascular malformations). On the other hand, regarding familial AVM, only two linkage analyses using 6 small families have been published by a research group [11,25], showing seven possible disease-responsible regions, i.e., 6q25 with the highest LOD score, 3p27, 4q34, 7p21, 13q32–q33, 16p13–q12 and 20q11–q13, but failed to identify the causative mutation. In sporadic brain AVM, microarray study showed that the *VEGFA*, *ITGA5*, *ENG* and *MMP9* genes that may involve vascular development or maintenance, are highly expressed in AVM compared with normal brain parenchyma [8,22,24].

Here we report results of a genome-wide linkage analysis on an AVM family with four affected members in two successive generations.

2. Materials and methods

2.1. Subjects

A Japanese family consisting of 19 members across four generations included two patients with brain AVM, one patient with pulmonary AVM and one patient with both brain and pulmonary AVM (Fig. 1). The proband (III-3) first exhibited intractable epilepsy at 13 years old and was diagnosed by magnetic resonance imaging (MRI) as having a brain AVM of 2 cm in diameter located in the right frontal lobe (Fig. 2). Chest X-ray at the first visit detected a nodular shadow in the right lower lung field, and a diagnoses of pulmonary AVM with a 24% of shunt-rate was made following angiogram made (Fig. 2). This was resected when the proband was 14 years old. The proband's brain AVM was treated by gamma knife surgery when she was 19 years old, followed by treatment with antiepileptic medication. Her mother (II-3) died of intracranial hemorrhage due to brain AVM, and the maternal grandfather (I-1) died of a cancer. Another patient (III-5) had asymptomatic brain AVM, which was accidentally diagnosed by MRI. His father (II-5) had pulmonary AVM instead of brain AVM. These four members were assigned to "affected", six members (II-6, III-1, III-6, III-7, IV-1, and IV-2) without AVM confirmed by MRI were "unaffected", and the remaining three (I-2, II-1, and IV-3) who were not assessed by MRI but had neither past history of recurrent epistaxis or gastrointestinal tract bleedings were "unknown". None of the members had any AVM-related diseases, such as HHT. Evaluation of cutaneous

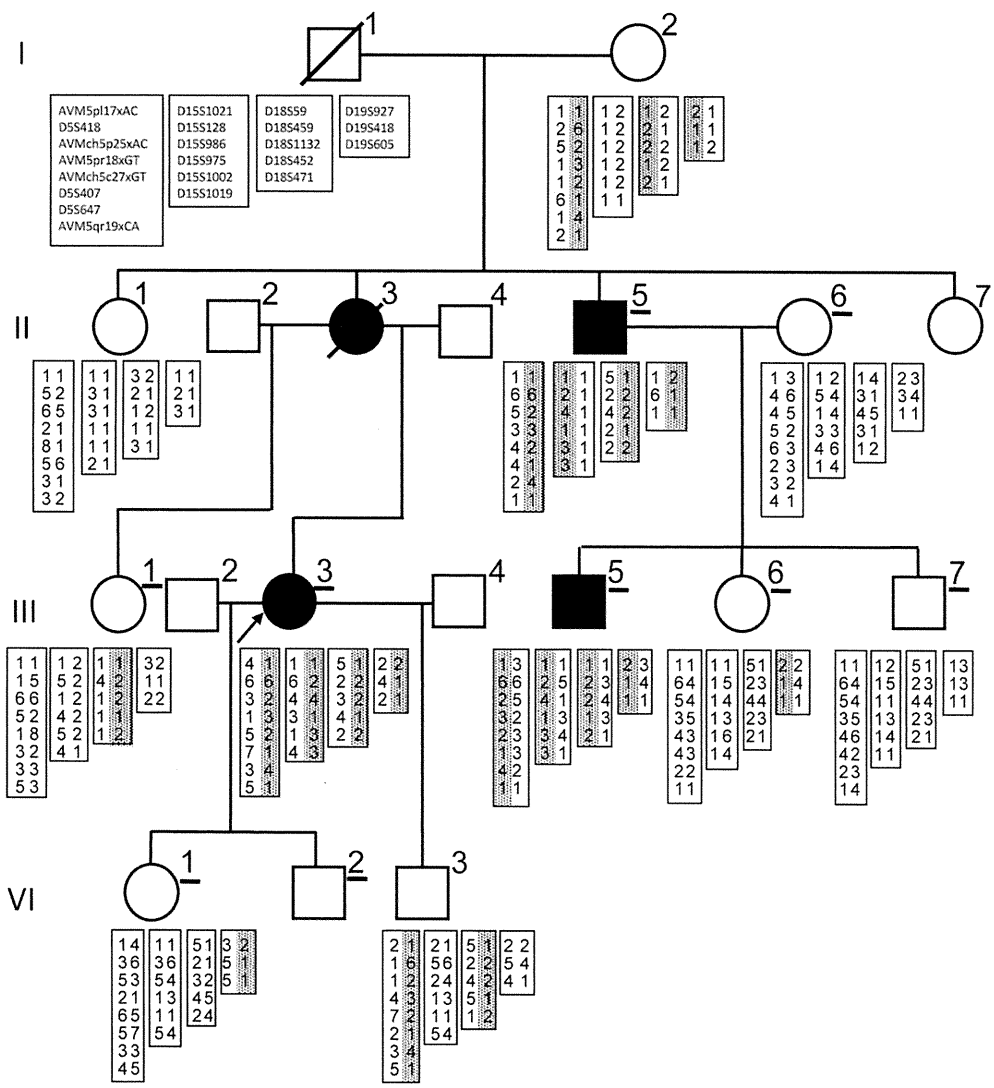

Fig. 1. Results of haplotype analysis at polymorphic loci in four regions, 5q13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.3–q13.42. Underlined individuals indicate those examined by MRI, and DNA was unavailable from individuals without haplotypes. Polymorphic alleles are numbered and candidate disease-associated haplotypes are shown by dotted boxes. Primer sequences designed for CA repeat amplification are available in Supplementary Table.

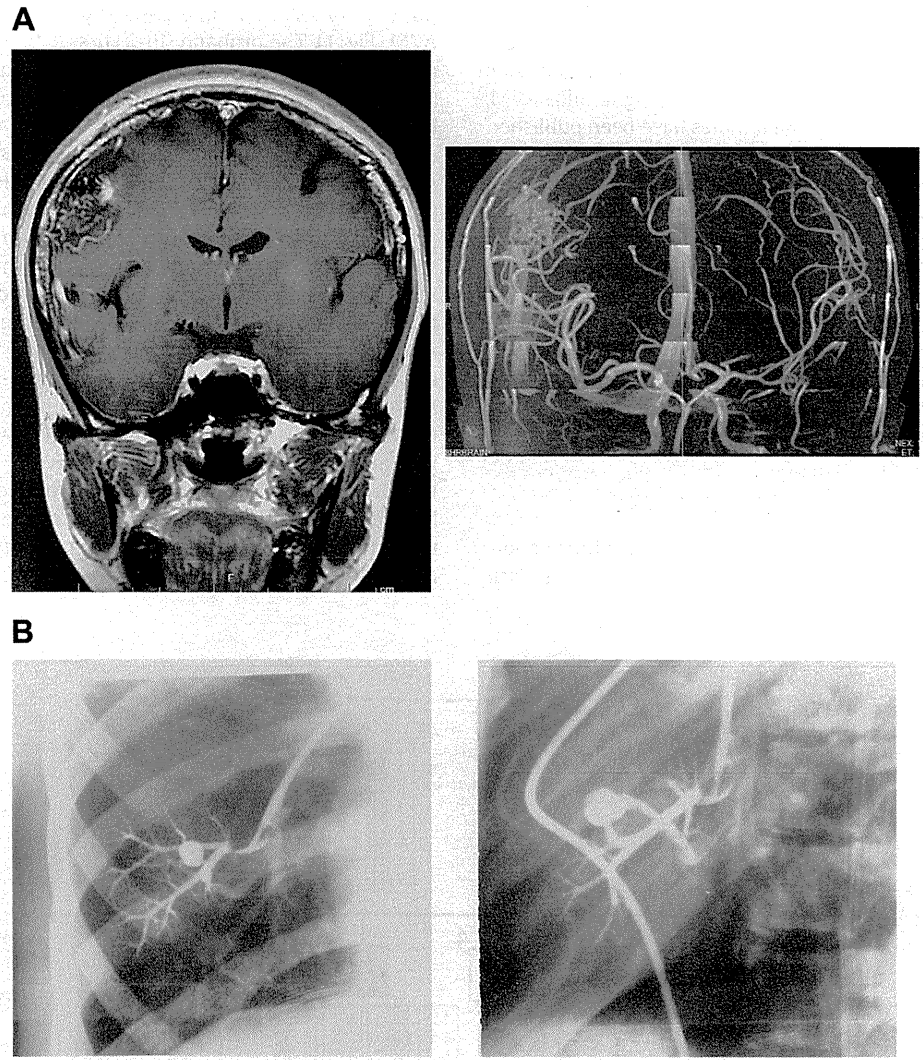

Fig. 2. Imaging of the brain and pulmonary AVM in the proband. (A) MRI scan and MR angiogram of the proband. The AVM is located right frontal lobe measured 2.0 × 1.3 cm. (B) Pulmonary angiograms of the proband. The pulmonary AVM is located in the right lower lobe (rtS8b) with 24% of shunt-rate.

lesions was conducted by examination of the proband and by detailed interview of the other family members by the proband and her sister (III-1), who is nurse. A total of 13 members participated in this study under informed consent. All experimental procedures for this study were approved by Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University.

2.2. DNA extraction

As a blood sample was available only from the proband, clipped fingernail samples were obtained from 10 of the other 12 members instead. Genomic DNA was extracted from the fingernails using a buffer solution containing urea, DDT and proteinase K, as reported previously [16,18]. Briefly, clipped fingernails were once frozen in liquid nitrogen and crushed into fine powder using Multi-beads Shocker™ (Yasui Kikai, Osaka, Japan). The nail powder was lysed in a urea-lysis solution (2 M urea; 0.5% SDS; 10 mM Tris–HCl, pH 7.5; 0.1 M EDTA) containing 1 mg/ml proteinase K and 40 mM DDT at 55 °C overnight. Nail DNA was extracted with phenol/chloroform, and precipitated with ethanol and sodium acetate. Precipitated nail DNA was dissolved again in extraction buffer (0.5% SDS; 10 mM Tris–HCl, pH 7.5; 0.1 M EDTA) containing 1 mg/ml proteinase K, and incubated at 55 °C overnight. DNA was purified again as above, and was suspended in 30 μl of 1× TE buffer.

2.3. SNP genotyping with Affymetrix 10K 2.0 array

Blood DNA (250 ng) was processed according to the standard protocol provided by the GeneChip Mapping 10K Xba Assay Kit (Affymetrix, Santa Clara, CA). Fingernail DNA was processed in a similar manner but with the two following modifications to adapt to the oligonucleotide microarray system [15]. Prolongation of digestion time from 120 min as the standard protocol to overnight; and increase of the PCR cycle number from 35 to 45 cycles. Data acquired from the Affymetrix GeneChip Operating System were analyzed using the Affymetrix GeneChip Genotyping Analysis Software (GTYPE) 4.0 to call genotypes.

2.4. Linkage analysis with SNP-genotype data and haplotype analysis with microsatellite markers

Multipoint LOD scores were calculated using MERLIN software [1], under an assumption that AVM in the family is transmitted in an autosomal dominant mode with reduced penetrance ($p = 0.9$)