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

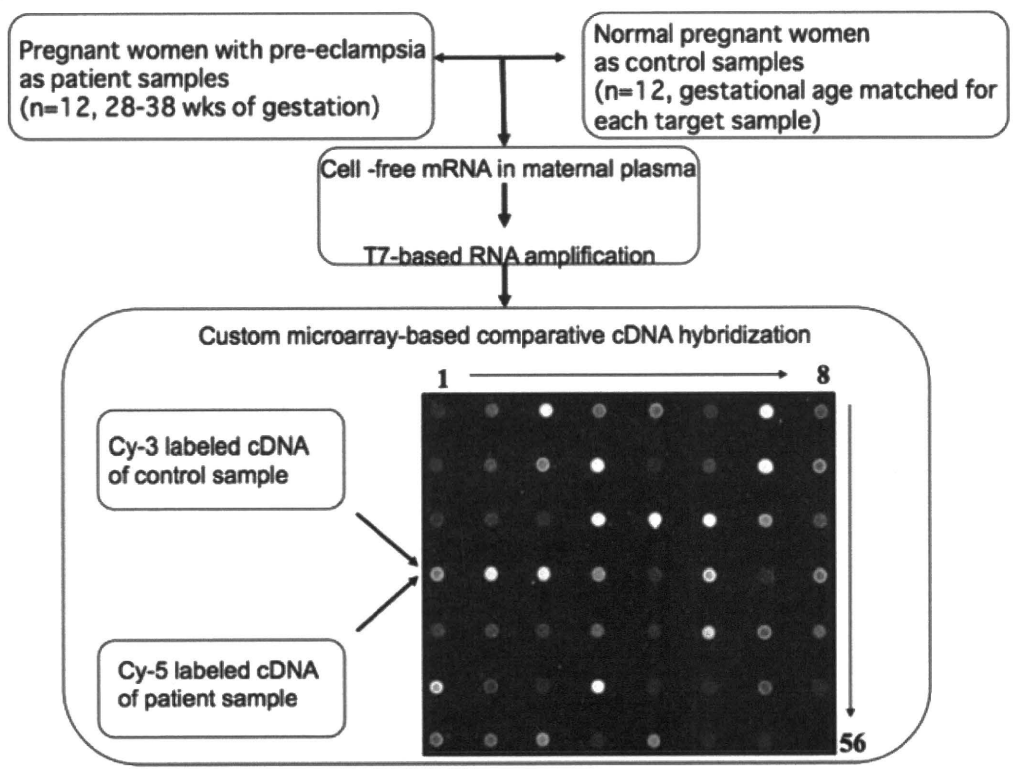

Figure 2—Outline of the custom microarray-based comparative cDNA hybridization. CF mRNA was extracted from 1.2 mL of individual plasma samples from 12 pregnant women with preeclampsia (27–38 weeks of gestation) and 12 normal pregnant women with matched gestational ages. The CF mRNA amplified by a T7-based 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.

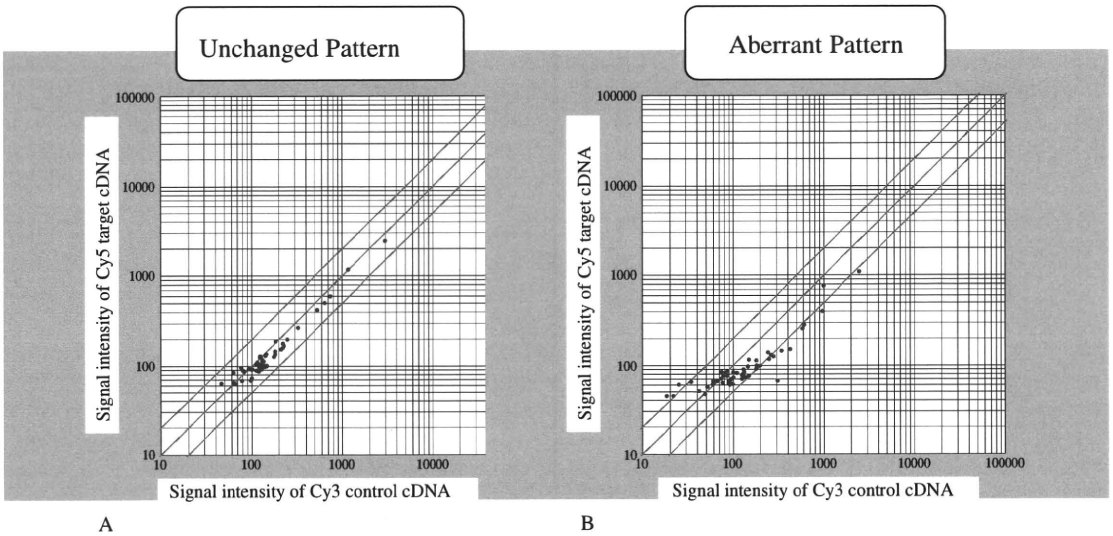

Figure 3—Scatter plots of the signal intensities obtained by comparative hybridization on the custom microarray between target cDNA from CF mRNA in plasma samples from preeclamptic women and control cDNA from normal control pregnant women. (a) Unchanged pattern showing that all the plots are within ±twofold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

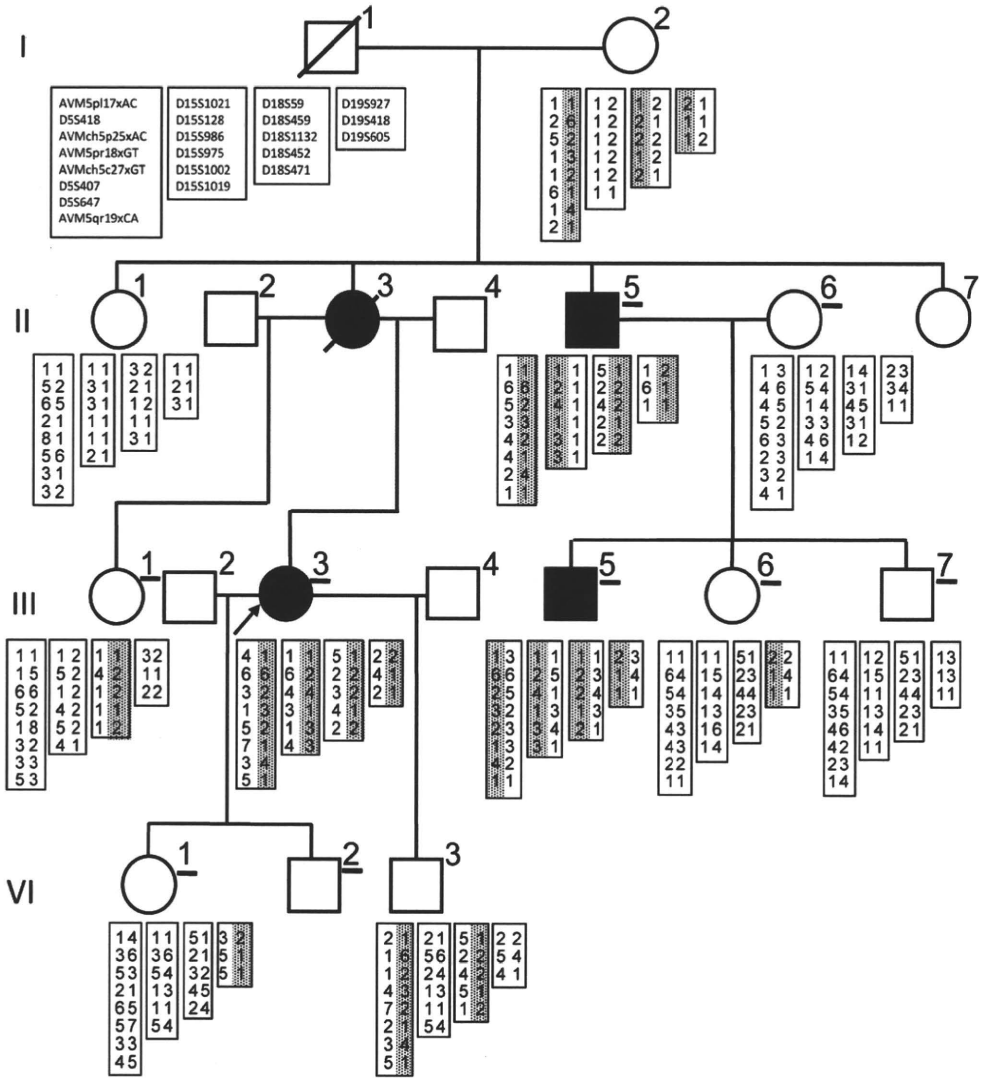

Fig. 1. Results of haplotype analysis at polymorphic loci in four regions, 5q13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.3–q13.42. Underlined individuals indicate those examined by MRI, and DNA was unavailable from individuals without haplotypes. Polymorphic alleles are numbered and candidate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

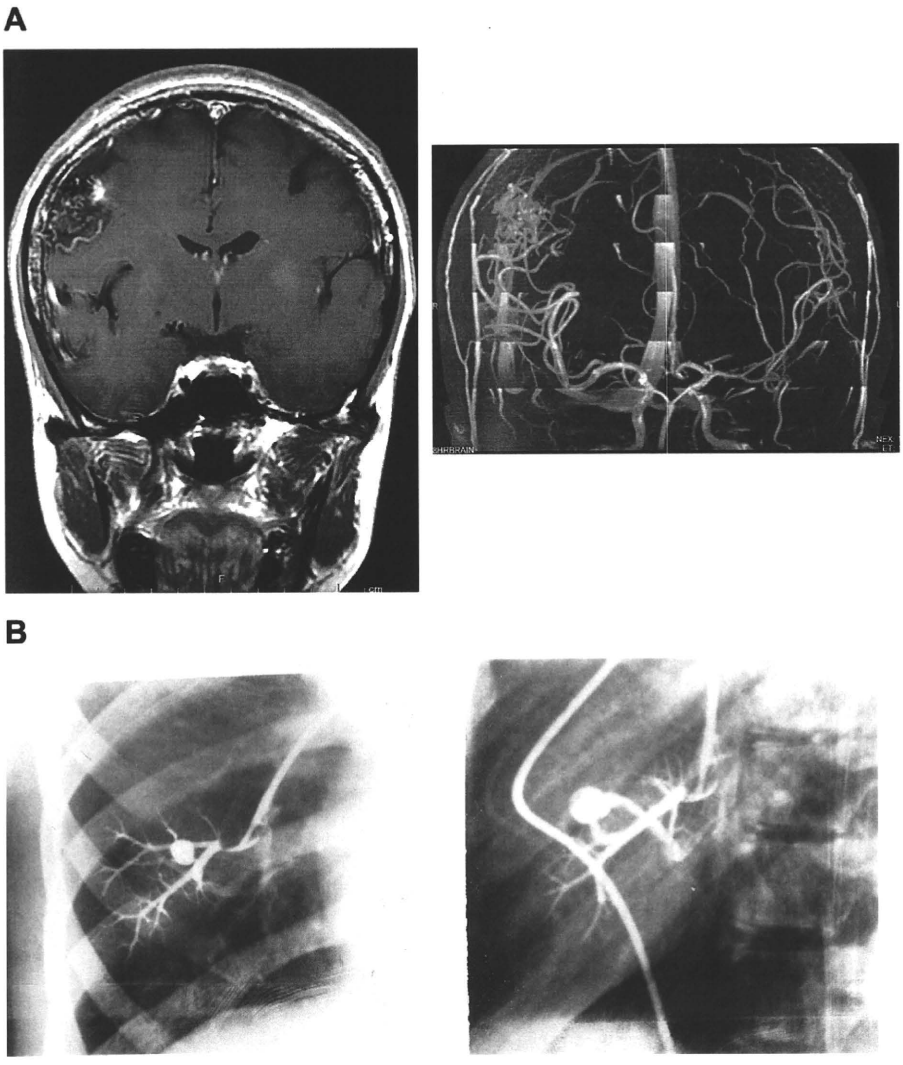

Fig. 2. Imaging of the brain and pulmonary AVM in the proband. (A) MRI scan and MR angiogram of the proband. The AVM is located right frontal lobe measured 2.0 × 1.3 cm. (B) Pulmonary angiograms of the proband. The pulmonary AVM is located in the right lower lobe (rtS8b) with 24% of shunt-rate.

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 ($p = 0.9$)

and with the disease allele frequency of 0.001. At loci with a positive LOD score by the GeneChip genotyping, possibly disease-associated haplotypes were constructed using SNP calls.

When SNP information was not informative, microsatellite markers were used for genotyping. Microsatellite markers used were referred to the National Center for Biotechnology Information (NCBI) database. One each of primer pairs for the markers was labeled with FAM, HEX, or NED (Supplementary Table 1), and PCR was performed in a 10 μl mixture containing 5 ng genomic DNA; 0.25 U ExTaq DNA polymerase HS-version (TAKARA Bio Inc., Kyoto, Japan); 200 μM dNTP; 0.5 μM primer; 1× ExTaq buffer on the T1 Thermocycler (Biometra, Goettingen, Germany). PCR products were separated on Genetic Analyzer 3130xl (AppliedBiosystems), and genotyping was carried out using GeneMapper software (AppliedBiosystems). At the regions where the affected individuals have a disease-associated haplotype, two-point LOD score was calculated by MLINK program (included in FASTLINK software version 4.0P) [14].

2.5. Mutation analysis

Some genes located within candidate regions identified by the linkage analysis were selected for further mutation analysis. A few other genes, albeit outside the regions, were also subjected to mutation analysis. Primer pairs for such genes were designed using Primer3-web 0.3.0 (http://frodo.wi.mit.edu/primer3/input.htm), according to their genomic sequences retrieved from the University of California, Santa Cruz (UCSC) Genome Browser Home (http://genome.cse.ucsc.edu/). PCR was carried out in a 15 μl reaction mixture containing 5 ng DNA; 0.25 U ExTaq DNA polymerase HS version; 200 mM dNTP; 0.5 μM each primer; 1× ExTaq buffer on the T1 Thermocycler. PCR products were subjected to direct sequencing, using BigDye Terminator v3.1 Cycle sequencing Kit (AppliedBiosystems) and Genetic Analyzer 3130xl. Electropherograms of sequences were aligned with ATGC software (GENETYX Corp., Tokyo, Japan) to inspect base alterations.

2.6. Search for genomic aberration

To search for copy number change within the candidate loci identified by linkage analysis, we used Affymetrix® Genome-Wide Human SNP Array 5.0 (920,568 probes; Affymetrix). Genomic DNA extracted from white blood cell of proband was processed according to manufacture's protocol. Intensity data from each probes were obtained from Affymetrix® Genotyping Console 3.0 as a CEL files. Unpaired copy number analysis of whole genome was carried out using Partek Genomics Suite (Partek, MO, USA) and regions with copy number change were determined by Hidden Markov Model at default settings.

3. Results

3.1. Linkage and haplotype analyses

The mean SNP call rate was 92.49% in 11 fingernail DNA samples, compared to 98.11% in a blood DNA sample from the proband. Incorrect SNP calls may result in seemingly inconsistent parent–child transmissions, but the call rates obtained are actually enough for further studies. We thus advanced to calculate LOD scores using these data.

The linkage analysis using MERLIN software revealed 18 regions with positive LOD scores (>0.00). Of the 18 regions, 14 with the following conditions were excluded: those without any functional full-length RefSeq genes; those in small size (<200 kb); and those in which some affected members did not have a common haplotype. Consequently, four loci, 5p13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.33–q13.42, remained as possibly linked regions (Figs. 1 and 3).

We then genotyped with microsatellite markers and calculated two-point LOD scores, considering the affected, unaffected, and the unknown family members. We confirmed three of the four candidate loci. They were a 48-Mb region between markers rs1366265 and rs1373965 at 5p13.2–q14.1, a 6-Mb region between rs850819

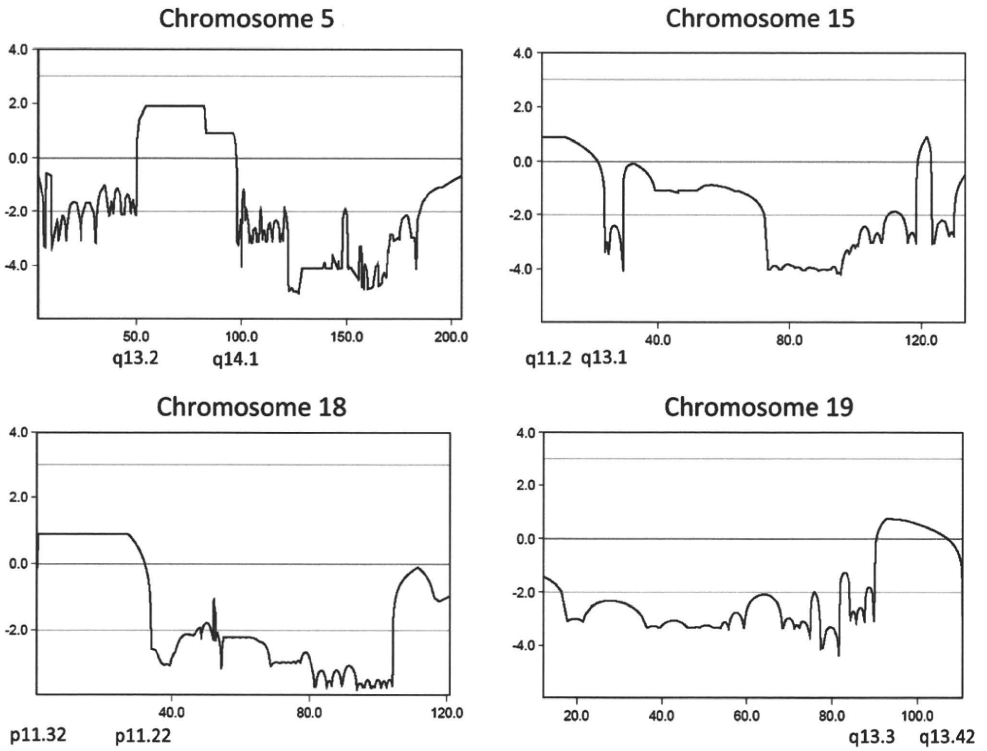

Fig. 3. Multipoint LOD scores calculated by MERLIN in four chromosomal regions, 5q13.2–q14.1, 15q11.2–q13.1, 18p11.32–p11.22 and 19q13.3–q13.42.

and rs818089 at 15q11.2–q13.1, both giving the maximum two-point LOD score of 1.632 ($\theta = 0$), and a 9-Mb region between rs486633 and rs1942150 at 18p11.32–p11.22 with the maximum LOD score of 0.851 ($\theta = 0$) (Table 1). As a possibly disease-associated haplotype on 19q13.33–q13.42 was transmitted to two definitively unaffected individuals (III-6 and IV-1), chromosome 19 was ruled out from the candidacy (Table 1, Fig. 1).

3.2. Mutation analysis of candidate genes

Within the 48-Mb region at 5p13.2–q14.1, there are about 200 RefSeq genes. Ten (*MAP3K1*, *DAB2*, *OCLN*, *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EDFLAM*, *ERBB2IP*, and *PIK3R1*) from these genes were focused and selected as candidates for brain AVM, since they concern development or maintenance of vessels, are associated with other heritable vascular disorders such as HHT, or are expressed in the brain with AVM [8,22,24]. Mutation analyses in these 10 genes revealed no pathologic mutation in the proband, although other affected members were not examined because of insufficient amount of their DNA. Although the genes endoglin isoform 1 precursor (*ENG*), activin A receptor type II like 1 (*ALK1*) and RAS p21 protein activator 1 (*RASA1*) are not located in the candidate region, we investigated whether any of them are involved in the etiology of AVM in the family as a partial symptom of HHT or AVM-CM. Direct sequencing of these three genes failed to show any causative variants.

Copy number analysis of proband revealed one increased copy number loci at 12q and decreased at 2p, 3q, 4q, 6p, 7q and 22q (data not shown). But all these alterations were reported previously as copy number polymorphisms (http://projects.tcag.ca/variation/) and out of our candidate loci. In addition, neither deletions nor microdeletions were detected at 9q34.11 of *ENG*, 12q13.13 of *ALK1* and 5q14.3 of *RASA1*.

4. Discussion

We have reported a family consisting of two affected members with brain AVM, one with pulmonary AVM and one with both brain and pulmonary AVM. The condition in this family met the criteria of familial brain AVM and seems to be inherited in an autosomal dominant mode. We tried to assign the location of a putative disease-gene by linkage analysis and search for mutations by subsequent candidate gene approach.

Table 1
Two-point LOD scores for brain AVM at various loci.

Locus	Recombination fraction (θ)					
	0.00	0.01	0.02	0.03	0.04	0.05
AVM5pl17xAC	0.032	0.030	0.029	0.027	0.026	0.025
D5S418	0.551	0.535	0.518	0.501	0.484	0.467
AVMch5p25xAC	1.334	1.301	1.268	1.234	1.201	1.167
AVM5pr18xGT	0.511	0.491	0.472	0.452	0.433	0.414
AVMch5c27xGT	1.630	1.597	1.564	1.531	1.497	1.463
AVM5c18xAC	1.373	1.344	1.314	1.285	1.255	1.225
D5S407	1.632	1.599	1.566	1.532	1.499	1.465
D5S647	1.154	1.121	1.089	1.056	1.023	0.991
AVM5qr19xCA	0.810	0.790	0.769	0.748	0.727	0.706
D15S1021	0.171	0.164	0.157	0.150	0.143	0.137
D15S128	0.876	0.858	0.841	0.823	0.805	0.787
D15S986	0.812	0.791	0.770	0.749	0.728	0.707
D15S975	0.400	0.387	0.374	0.361	0.348	0.335
D15S1002	1.330	1.298	1.266	1.234	1.202	1.170
D15S1019	1.632	1.599	1.566	1.532	1.499	1.465
D18S59	0.199	0.214	0.225	0.234	0.241	0.246
D18S459	0.142	0.136	0.131	0.125	0.120	0.114
D18S1132	0.677	0.663	0.650	0.636	0.623	0.609
D18S452	0.851	0.832	0.813	0.794	0.774	0.755
D18S471	0.240	0.231	0.222	0.214	0.205	0.197
D19S927	−0.302	−0.277	−0.254	−0.234	−0.216	−0.200
D19S418	−2.655	−2.453	−2.257	−2.078	−1.919	−1.778
D19S605	−0.648	−0.574	−0.512	−0.460	−0.414	−0.374

The linkage analysis of the family revealed three candidate regions (5p13.2–q14.1, 15q11.2–q13.1, and 18p11.32–p11.22) with relatively high LOD scores of 1.632, 1.632 and 0.851, respectively (Table 1). However, neither region was conclusive. This insufficient mapping may have arisen from the small pedigree size, and/or from incomplete ascertainment of affected members, e.g., probable existence of asymptomatic affected persons among the "unknown" members. Indeed, as for a candidate locus at 5p13.2–q14.1, the proband's maternal grandmother (I-2) and son (IV-3) had a haplotype common to the three affected members (Fig. 1), but they were fallen into the "unknown" individuals. If DNA from IV-2 was available and if MRI examinations of VI-3 and I-2 were carried out, we would have obtained more definitive results. As we performed linkage analysis using high-density SNP genotyping, 14 small regions not containing RefSeq genes or miRNAs showed a positive LOD score. It is possible that an unidentified transcribed RNA in one of these regions could cause familial AVM, but these regions are candidate loci with a lower priority than those containing known genes. Thus, the three regions have remained at present as the equally possible loci for AVM. The three regions do not overlap with a previously reported candidate locus of familial brain AVM, i.e., 6p25 [11], and do not contain genes responsible for syndromic AVM (heritable disorders involving AVM) or cerebral cavernous malformations, such as *ENG* [17], *ALK1* [12], *RASA1* [3,6,20,21,26], and *PTEN* [23], *KRIT1* [13], *MGC407* [5], *PDCD10* [2].

We then searched for mutations in 10 genes within 5p13.2–q14.1, among which *MAP3K1*, *DAB2* and *OCLN* encode proteins playing roles in the TGF-β signaling pathway, and *FGF10*, *ESM1*, *ITGA1*, *ITGA2*, *EGFLAM*, *ERBB2IP* and *PIK3R1* were those expressed in brain AVM tissues by previous microarray analysis [8,22,24]. Nevertheless, no pathologic mutation was found in any of them. Because the presence of both brain AVM and pulmonary AVM in this pedigree is reminiscent of Hereditary Hemorrhagic Telangiectasia, we analyzed *ENG* and *ALK1* for mutations and genomic aberrations, which may cause HHT1 and HHT2 respectively [12,17]. The proband did not have any mutations in the coding exons or intron/exon boundaries of either gene, nor any genomic aberrations at those loci. We also analyzed *RASA1* because this may cause CM-AVM, which is characterized by multiple CM and AVM [3,6,20,21,26]. No causative mutation or genomic aberration was detected in the proband. Although other genes, such as *KRIT1*, *MGC407* and *PDCD10*, have been shown to cause slow-flow lesions i.e., cerebral cavernous malformation [2,5,13], they were not investigated in the present study, because the clinical manifestations in our family did not meet the criteria for these diseases.

Participation of family members and compliance with guidelines for human genome researches are critical to conduct a linkage analysis. Whole-blood samples cannot occasionally be available in some family members because of their far domicile. In such the case, fingernail DNA is useful, since clipped fingernails can be mailed in a usual way, and stored long at a room temperature, as indicated previously [16,19]. The present study is the first experience to adopt fingernail DNA to genome-wide high-density SNP microarray analysis. The performance obtained from fingernail DNA was sufficient, showing all SNP call rates of >86%. According to the manufacture's protocol, samples with an SNP call rate of <85% should further be evaluated before including the data in downstream analysis. Incorrect SNP calls may make serious problems in linkage analysis. For instance, SNPs with parent–child transmission inconsistency may be omitted, leading to a reduced LOD score.

In conclusion, we have assigned the familial AVM locus to three alternative regions, 5p13.2–q13.2, 15q11.2–q13.1 and 18p11.32–p11.22, by a genome-wide, high-density, SNP-based

linkage analysis with fingernail DNA in an AVM family. However, mutation analyses of some genes in the regions failed to identify any pathological changes.

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Competing interests

There are no competing interests.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmg.2010.06.007.

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Failure to Confirm CNVs as of Aetiological Significance in Twin Pairs Discordant for Schizophrenia

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Copy number variations (CNVs) are common structural variations in the human genome that strongly affect genomic diversity and can play a role in the development of several diseases, including neurodevelopmental disorders. Recent reports indicate that monozygotic twins can show differential CNV profiles. We searched CNVs in monozygotic twins discordant for schizophrenia to identify susceptible loci for schizophrenia. Three pairs of monozygotic twins discordant for schizophrenia were subjected to analysis. Genomic DNA samples were extracted from peripheral blood lymphocytes. We adopted the Affymetrix Genome-Wide Human SNP (Single Nucleotide Polymorphism) Array 6.0 to detect copy number discordance using Partek Genomics Suite 6.5 beta. In three twin pairs, however, validations by quantitative PCR and DNA sequencing revealed that none of the regions had any discordance between the three twin pairs. Our results support the hypothesis that epigenetic changes or fluctuation in developmental process triggered by environmental factors mainly contribute to the pathogenesis of schizophrenia. Schizophrenia caused by strong genetics factors including copy number alteration or gene mutation may be a small subset of the clinical population.

Keywords: CNVs, schizophrenia, genotype, monozygotic twin, epigenetic change

Schizophrenia is a chronic, debilitating psychiatric illness with a 1% worldwide prevalence. Genetic studies have shown that schizophrenia has a high heritability, with strong genetic factors involved in its etiology. Twin studies have played an important role in the elucidation of the genetic factors underlying neurodevelopmental disorders. Several twin studies have revealed that the concordance rate between monozygotic twins is 41–79% for schizophrenia, whereas the concordance rate between dizygotic twins is 0–14% (Shih et al., 2004; Kakiuchi et al., 2008). The higher concordance rate in monozygotic rather than dizygotic twins for schizophrenia suggests the contribution of genetic factors. Phenotypically discordant monozygotic twins are especially interesting resources for genetic studies, and twin studies could facilitate the identification of the causative genes of phenotypes. Kondo et al. (2002) reported that a nonsense mutation in *IRF6*, which is a causative gene for Van der Woude syndrome, was found in one affected individual in monozygotic phenotypically discordant twins. In relation to neurodevelopmental disorders, Bruder et al. (2008) reported that discordant monozygotic twins with parkinsonism showed different copy number variation (CNV) profiles.

CNVs are the most prevalent type of structural variations in the human genome that largely contribute to genomic diversity. Redon et al. (2006) and Carter et al. (2007) showed that as much as 12% of the human genome and thousands of genes are variable in copy number. A great number of CNVs may not be pathogenic but simply contribute to human genome diversity not related to phenotype. Meanwhile, some CNVs have been proven a significant factor related to disease susceptibility. Recent studies reported that CNVs contribute to genetic vulnerability factors and can play an important role in the etiology of several neurodevelopmental disorders (Sebat et al., 2007; 2009). Xu et al. (2008) found that de novo copy number mutations were about eight times more frequent in patients with sporadic schizophrenia. Numerous copy number analyses in schizophrenia revealed that genes that were disrupted by CNVs, which include *TBX1*, *ERBB4*, *SLC1A3*, *RAPGEF4*, *CIT*, *NRXN1*, and 16p11.2 region, were candidate genes and regions for schizophrenia (Cook et al., 2008; McCarthy et al., 2009; Merikangas et al., 2009; Walsh et al., 2008); however, most of these are rare copy number variants and the contribution of those genes to schizophrenia is restricted to a tiny part of etiologies.

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To date, numerous causative genes for schizophrenia have been identified; however, because of genetic heterogeneity, there is still a long path to the elucidation of the pathogenesis of schizophrenia. To identify causative genes for schizophrenia, we have utilized the Affymetrix Genome-Wide Human SNP Array 6.0 in three pairs of monozygotic twins discordant for schizophrenia. Here, we describe the results of CNV and genotype profiles in three pairs of monozygotic twins.

Methods

Subjects

Three pairs of monozygotic twins discordant for schizophrenia participated in this study. Ten years had passed after the onset of schizophrenia in the affected individuals in all twin pairs. All of the twins were male, and their mean age was 53 years old. Two well-trained psychiatrists diagnosed the twins by structured clinical interview, and all affected individuals corresponded to the DSM-IV-TR criteria for the undifferentiated type of schizophrenia.

DNA Microarrays

Ten ml of peripheral blood samples was collected after obtaining written informed consent, and genomic DNA was extracted from blood lymphocytes using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Experimental procedures were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis at Nagasaki University.

DNA microarray experiments were performed using Affymetrix Genome-Wide Human SNP Array 6.0 (SNP Array 6.0) (Affymetrix, Santa Clara, CA, USA). We performed a paired analysis for loss of heterozygosity (LOH) and an unpaired analysis for copy number analysis using control individuals' data. All of the computer analyses were performed using Genotyping Console (Affymetrix) and Genomics Suite version 6.5 beta software (Partek, St. Louis, MO, USA). Genomic copy number data were analyzed with Partek Genomics Suite software using a segmentation algorithm with stringent p value cutoff.

Quantification of Genome Copy Number

We performed real-time quantitative PCR using an intercalating dye, SYTO13 (Molecular Probes, Eugene, OR, USA), which is an alternative to SYBR green I, or using Universal Probe Library (Roche Diagnostics, Mannheim, Germany) to verify copy number changes suggested by the microarray analyses. Primers and probes were designed using the website software Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com). Real-time PCR amplification was run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). All samples were measured in tetraplicates.

DNA Sequencing

To verify the SNP genotypes obtained by SNP Array 6.0, we performed direct sequencing of PCR-amplified genomic DNA fragments including SNPs that showed discordant allele calls in each twin pair. The amplified fragments were directly sequenced after purification with exonuclease I and NTPhosTM Thermolabile Phosphatase (Epicentre, Madison, WI, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit and run on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed using Variant Reporter (Applied Biosystems) and ATGC version 6.0 (Software Development, Tokyo, Japan).

Results

Microarray Analysis Results

Quality control (QC) data obtained from the SNP Array 6.0 are summarized in Table 1. The call rate and contrast QC in SNP Array 6.0 data were > 95% and > 1.50, respectively, for all samples, and both values indicated experiments using the SNP Array 6.0 were done well.

Copy number analysis of microarray data using Partek Genomics Suite showed some deleted or amplified regions in each twin pair (data not shown). Regions with discordant genotyping between twins from microarray data are summarized in Table 2.

Unpaired analysis of 6 individuals in comparison with ethnically-matched normal controls (HapMap-JPT) revealed that an approximately 3 kb region within the *SLC25A37* gene was deleted in two of the three schizophrenia twin pairs, 11A/B and 31A/B. The deleted region (chromosome 8:23460969–23463786) was not registered in the Database of Genomic Variants (http://projects.tcag.ca/variation/).

Quantitative PCR Results

We verified the copy number state by real-time PCR of the regions with discordant copy number, including genes, by paired analysis using SNP Array 6.0. Primers were designed for the middle position of the regions.

Table 1

Summary of Twin Samples and Affymetrix GeneChip Genotyping Results

Samples	Sex	Phenotype	SNP call rate	Contrast QC*
11A / B	Male	Schizophrenia/unaffected	99.444 / 99.516	2.38 / 2.48
21A / B	Male	Schizophrenia/unaffected	98.974 / 99.175	1.88 / 2.22
31A / B	Male	Schizophrenia/unaffected	99.199 / 99.179	2.26 / 1.60

Note: *Contrast QC (Quality Control) is per sample Quality Control test metric for SNP Array 6.0 intensity data. In high-quality data sets, the Contrast QC metric is higher than the 0.4 threshold according to user manual provided by the manufacturer.

Table 2

List of Loss of Heterozygosity Regions Derived from Microarray Data

chr. #	Physical position		Twin #	Validated SNPs	Overlapping genes
	Start	End			
1	4309356	4465925	11A / B	rs7521665, rs4654438	LOC284661
	45006976	45050681	31A / B	rs6676749	BEST4, PLK3, RPS8, SNORD38A, SNORD38B, SNORD46, SNORD55
	170792582	170870563	31A / B	rs2472550	Region overlaps with 70.55% of C1orf9
2	50182138	50311147	31A / B	rs1452762, rs6712119	Contained within NRXN1
	142093343	142097262	31A / B	rs355581	Contained within LRP1B
3	3693732	3821526	31A / B	rs7613060, rs769806	Region overlaps with 4.23% of LRRN1
	123371895	123393318	31A / B	rs1501900	Region overlaps with 37.81% of CASR
4	24093201	24162064	31A / B	rs4697063	Region overlaps with 34.68% of DHX15
	81368193	81418990	11A / B	rs10518238, rs1458046	Region overlaps with 24.07% of FGF5
	101451872	101646851	31A / B	rs3756037	Region overlaps with 57.10% of EMCN
	109080142	109167540	31A / B	rs4395588	Region overlaps with 15.93% of CYP2U1 and 42.51% of HADH
	126258785	126764905	31A / B	rs7660602	FAT4
5	38382422	38389445	11A / B	rs9292705	Contained within EGFLAM
	166816487	166823787	31A / B	rs17068499	Contained within ODZ2
6	35297977	35376388	31A / B	rs3800385	ZNF76, region overlaps with 3.59% of DEF6 and 36.49% of SCUBE3
	119363250	119468737	31A / B	rs6913082	Contained within FAM184A and 74.19% of FAM184A
8	86383578	87077669	31A / B	rs1845891, rs1553015, rs6605618	CA1, CA2, CA3, REXO1L1, REXO1L2P
9	207826	208183	31A / B	rs10964703	Contained within DOCK8
	3900136	3920251	31A / B	rs630219	Contained within GLIS3
	7154039	7156090	31A / B	rs1556100	Contained within KDM4C
	112777053	112781741	31A / B	rs3758281, rs16915618	Contained within LPAR1
10	68497020	68657339	31A / B	rs10822972	Contained within CTNNA3, region overlaps with 21.12% of LRRTM3
	100181485	100219522	31A / B	rs11599112	Region overlaps with 28.02% of HPSE2 and 39.99% of HPS1
11	8896463	9040536	11A / B	rs4929922	C11orf17, region overlaps with 29.17% of SCUBE2
	19449860	19466526	31A / B	rs11820210	Contained within NAV2
12	21894811	21895465	31A / B	rs4148673	Contained within ABCC9
	33716220	36801139	31A / B	rs11052835, rs2387324	ALG10
	38818800	39404433	21A / B	rs7132869	LRRK2, region overlaps with 5.43% of CNTN1
	63692809	63739310	11A / B	rs4964104	Region overlaps with 18.58% of WIF1
	69385261	69392041	31A / B	rs10879183	Contained within PTPRR
	77123022	77139445	31A / B	rs9971904	Region overlaps with 48.10% of NAV3
	120088239	120155175	31A / B	rs25643	Region overlaps with 29.88% of P2RX7 and 34.55% of P2RX4
13	102227169	102252370	31A / B	rs9514058	KDELC1, region overlaps with 11.79% of BIVM
16	13150832	13161027	31A / B	rs4781419	Contained within SHISA9
22	24570234	24607029	31A / B	rs6004793	Contained within MYO18B
	36847351	36893417	31A / B	rs2076116	Contained within PLA2G6

Note: Chr. # means the number of chromosome.

Quantitative PCR was performed for a total of 120 regions. However, we could not reconfirm the differences between twins in all 120 tested regions. In addition, quantitative PCR within the *SLC25A37* gene revealed no loss or gain of the genome in comparison with ethnically matched control individuals.

Sequencing Results

DNA sequencing was performed for a total of 37 regions surrounding SNPs that had shown discordant genotype calls from microarray analysis within twin pairs. We selected one or more SNP(s) called discordant genotype in each LOH region. Sequencing revealed all of the SNPs were concordant between twin pairs (data not shown).

Discussion

In this study, we analyzed genomic alterations, CNVs and genotypes, in three pairs of monozygotic twins discordant for schizophrenia. None of the regions of copy number difference between twins shown by SNP Array 6.0 were reverified by quantitative PCR, and none of the genotype discordance was reverified by sequencing. Additionally, no novel CNVs was detected in the identified CNVs between twins. To our knowledge, this is the first report verifying the data from high-density and high-resolution DNA microarrays by quantitative PCR and DNA sequencing. Our results indicate that genomic alterations including CNVs and gene mutations contribute minimally to etiologies of

schizophrenia. The large genome-wide study by The Wellcome Trust Case Control Consortium (WTCCC) revealed CNVs is not main cause of bipolar disorder, which is one of the neurodevelopmental disorders (WTCCC, 2010). This report have a different concept from our study because our study aimed to find copy number alteration as a single gene disorder, however, WTCCC report could not discover the CNV contributing to the bipolar disorder. Our results may support the hypothesis that epigenetic changes (Roth et al., 2009), which can influence the expression of genes without affecting the DNA sequence, mainly contribute to the pathogenesis of schizophrenia.

SNP Array 6.0 allows us to detect different genotype or copy number neutral LOH regions. In our twin comparison, copy number neutral LOH would indicate segmental uniparental disomy (UPD) in twin pairs. Actually, UPD of the paternal allele at 11p15 in the affected twin caused discordance for hemihypertrophy in monozygotic twins (West et al., 2003). Furthermore, recent studies revealed that UPD was associated with schizophrenia. UPD on chromosome 1 and 5q32-qter in a patient with schizophrenia has been described in 2004 and 2006, respectively (Abecasis et al., 2004; Seal et al., 2006). But no genotype difference between twins was confirmed in this study.

Here, we presented no genomic discordance between twins; hereinafter, we will discuss some speculation about the relation between genetic factors and phenotypic discordance. First, it is possible that mosaicism at specific tissues (i.e., brain) because of postzygotic genomic rearrangements causes discordant phenotypes between monozygotic twins. Although we used DNA samples extracted from peripheral blood cells in this study, mosaic genomic rearrangement could be detected in brain. It is clear that the ideal source for studies of neurodevelopmental disorders is brain tissue. Nonetheless, it is practically impossible to harvest the brain tissue of twins (Kato et al., 2005). Olfactory sensory neurons have recently been shown to be easily accessible neuronal cells that have been useful for studies on schizophrenia (Arnold et al., 2001), enabling the study of neuronal cell character including genotype and copy number state. Second, it is possible that smaller-scale genomic aberrations below detection sensitivity influence the discordant phenotype of monozygotic twins. SNP Array 6.0 is one of the highest resolution platforms commercially available and allows us to identify CNVs much smaller than 10 kb. However, McCarroll et al. (2008) showed that the detection rate using the SNP Array 6.0 sharply diminished for CNVs <4 kb. To increase sensitivity, the use of many more detection probes is needed, and more than one experimental platform should be performed in future studies.

Bruder et al. (2009) successfully detected many copy number changes in peripheral blood using a Bacterial Artificial Chromosome (BAC)-array at mosaic state (~20%) in nine monozygotic twins discordant for parkinsonism. All of the nine pairs had copy number discordancy in their reports. Because their results suggested copy number change could be found in the mosaic state, tissue-specific mosaicism is a possible explanation for psychiatric disorders. We may have overlooked copy number change in a mosaic state in peripheral blood with the use of the SNP Array 6.0 instead of the BAC-array because the SNP Array 6.0 is a powerful tool to identify small regions with copy number change but is not suitable to detect copy number in a mosaic state.

It seems most likely that epigenetic changes between monozygotic twins influence the phenotypic discordance of monozygotic twins. Several recent studies indicate that epigenetic changes contribute to the etiology of schizophrenia. Rett syndrome and Fragile X syndrome are neurodevelopmental disorders caused by a single gene defect and dysregulation of DNA methylation very early in life (Amir et al., 1999; Das et al., 1997). Kaminsky et al. (2009) have shown that differences in DNA methylation profiles increase in monozygotic twins along with aging. Because the onset of schizophrenia is later than Rett syndrome and Fragile X syndrome, it is possible that cumulative epigenetic modifications could be one cause of schizophrenia development. Furthermore, a recent study by Roth et al. (2009) suggested that DNA methylation and histone modification triggered by influence of environmental factors is responsible for the difference in onset age between these disorders. Akbarian et al. (2005) indicated that histone modification contributes to the pathogenesis of prefrontal dysfunction in patients with schizophrenia based on the finding that the level of H3-(methyl)arginine 17 in patients with schizophrenia exceeded control values by 30%. Thus, genome-wide DNA methylation and genome-wide histone modification studies for monozygotic twins discordant for phenotypes may be promising techniques in future twin studies. In fact, Baranzini et al. (2010) reported genomic sequence and epigenetics (methyl-cytosine) analysis of monozygotic twin discordant for multiple sclerosis using next generation sequencer. They could not find reproducible differences between twins, but these comprehensive analyses including genome and epigenome sequence are just started. As Crow (2002) discussed, it is important to analyze the genetic and epigenetic influence to the species-specific characteristics. Comprehensive genetic and epigenetic analysis of discordant monozygotic twins will be advanced using next generation technologies.

In summary, we did not detect genomic alterations including CNVs and gene mutations between twins discordant for phenotype. Our results indicate that schizophrenia caused by genomic alterations may be a small subset of the clinical population and may support the hypothesis that epigenetic mechanisms triggered by the influence of environmental factors are associated with the etiology of schizophrenia. Experimental investigations of epigenetic mechanisms

such as expression analysis, methylation site sequence and histone modification studies using DNA samples extracted from olfactory sensory neurons are needed to identify the differences responsible for discordant phenotypes in future studies.

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