MHC. INDO is a 42 kDa cytosolic monomeric protein, which catalyzes the degradation of the indole ring of tryptophan and other indoleamines (Hayaishi, 1985). The INDO gene is located on chromosome 8 (8p12–p11) and contains 10 exons (Kadoya et al., 1992).

Although INDO was first described in 1963 and early attention focused on its role in antimicrobial resistance, the biological significance of INDO has now been examined (Taylor and Feng, 1991; Hayaishi, 1993; Mellor and Munn, 1999). The expression of INDO by dendritic cells (DCs), monocytes and macrophages results in immunomodulatory effects on T cells due to the peri-cellular degradation of the essential amino acid tryptophan (Munn et al., 1999). In a comprehensive review Grohmann et al. (2003) discussed the roles of INDO in the control of T cell activity during infection, pregnancy, autoimmunity, transplantation and neoplasia (Grohmann et al., 2003).

Recurrent spontaneous abortion (RSA) is defined as "occurrence of three or more consecutive pregnancy losses before 20 weeks of gestation, with a fetus weighing 500 g or less" (WHO Recommended Definitions, 1997; Garcia-Enguidanos et al., 2002). It is believed that approximately one in 300 women globally experiences RSA. Although various etiologic factors have been postulated, the exact underlying pathophysiologic mechanisms remain elusive in up to 40–50% of cases (Philipp et al., 2003).

In this investigation, we hypothesized that INDO gene polymorphisms and allele frequencies are different in RSA patients to normally fertile control women. To test this hypothesis, we screened INDO gene exons and adjacent intronic regions for SNPs and evaluated their frequencies in RSA patients and in matched control women.

2. Materials and methods

2.1. Subjects

The subjects consisted of 111 southern Iranian women (aged 17–38 years; mean 27.2 years) who had experienced at least three RSA (mean 3.5) and in whom anatomical, hormonal, chromosomal, infectious and autoimmune causes including anti-phospholipid syndrome, had been excluded. They all attended the Department of Obstetrics and Gynecology Clinic of Shiraz University of Medical Sciences. The control individuals consisted of 105 ethnically matched women (aged 22–50 years; mean 36.5 year) who had at least two children (mean 3.4) and no history of pregnancy loss. The subjects and controls participated in this study after informed consent.

2.2. DNA extraction and sequencing of INDO

Venous blood was collected in EDTA-coated tubes, and DNA extracted using the salting out method (Miller et al., 1988). For detection of new single nucleotide polymorphisms (SNPs) we sequenced DNA samples from 111 RSA women for ten exons and adjacent intronic regions of the INDO gene. Forward and reverse primers specific for each exon (Nippon Gene Co. Ltd., Japan) were used in PCR in a mixture containing 25 ng DNA as template. PCR products (10 μl) were cleaned up using Exo SAP-IT enzymatic solution (Usb Corp., USA). Cleaned up PCR products were used as template in sequencing reactions. Sequencing primers (Nippon Gene Co. Ltd., Japan) and Big Dye terminator V3.1 cycle sequencing kit (PE Applied Biosystem, USA) were used in sequencing reactions according to the manufacturer's protocol. After preparation, samples were analyzed using an ABI PRISM 3100 machine. Resultant electropherograms were analyzed by DNA sequencing analysis software version 3.7 (PE Applied Biosystem). For detection of SNPs, electropherograms were aligned using of Auto-assembler version 2.1 (PE Applied Biosystem).

2.3. Statistical analysis

The frequency of each polymorphic allele was calculated by the allele counting method. Differences in the genotype and allele frequencies between patients and controls were tested by χ^2 analysis. Haplotype estimation and differences in the haplotype frequencies between RSA cases and control group were analyzed by Arlequin version 2000 software (http://anthro.unige.ch/arlequin).

3. Results

3.1. Sequencing analysis

3.1.1. SNP identification

Sequencing of ten exons and adjacent intronic regions of INDO in 111 RSA women detected 10 different base changes. These included one deletion (IVS3+562 del C) at the upstream region of exon 4, one SNP [325 G→A (Val 109 Ile)] within exon 4, three SNPs (IVS6+32 T→G, IVS6+54 T→A and IVS6+61 G→A) in intron 6, one SNP (IVS8+116

Table 1
Detected SNPs in screening of exons and adjacent intronic regions of *INDO* in RSA patients using automated sequencing.

SNPs	NCBI SNP database	Position	Allele frequency (allele)
IVS3+562 del C	rs 4259403	Intron 3	0.88 (C)
325 G→A (Val 109 Ile)	[a]	Exon 4	0.995 (G)
IVS6+32 T→G	[a]	Intron 6	0.995 (T)
IVS6+54 T→A	[a]	Intron 6	0.99 (T)
IVS6+61 G→A	[a]	Intron 6	0.97 (G)
IVS8+116 T→G	rs 9298586	Intron 8	0.995 (T)
720 C→T (Asp 240 Asp)	[a]	Exon 9	0.995 (C)
805 G→A (Val 269 Ile)	[a]	Exon 9	0.995 (G)
IVS9+2431 G→A	rs 3739319	Intron 9	0.42 (G)
954 G→A (Glu 318 Glu)	[a]	Exon 10	0.98 (G)

[a] The seven novel SNPs.

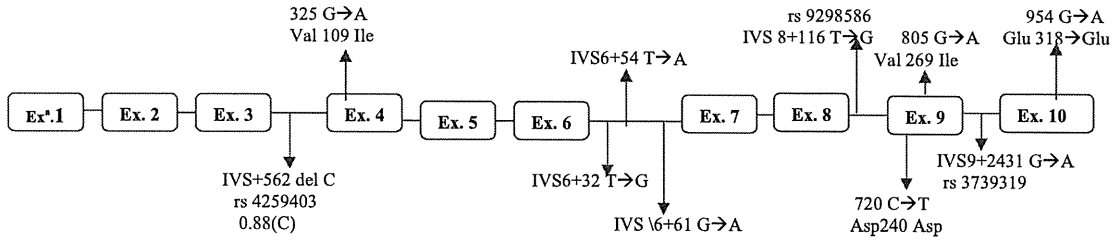

Fig. 1. Schematic illustration of the positions of detected SNPs region of *INDO* in RSA patients using automated sequencing. Ex[a]: exon.

T→ G) in intron 8, two SNPs [720 C→ T (Asp 240 Asp) and 805 G→ A (Val 269 Ile)] within exon 9, one SNP (IVS9+2431 G→ A) in intron 9 and one SNP [954 G→ A (Glu 318 Glu)] in exon10. Three of the detected SNPs had been registered in the Pubmed SNP database (Table 1 and Fig. 1). The one SNP at intron 6 (IVS6+32 T→ G) was only seen in the control group. Allelic frequencies of two intronic SNPs in intron 3 and 9 were 12% and 56% respectively. Also we detected two nonsynonymous SNPs in exon 4 (325 G→ A Val 109 Ile) and exon 9 (805 G→ A Val 269 Ile); and two synonymous SNPs, one in exon 9 (720 C→ T Asp 240 Asp) and the other in exon 10 (954 G→ A Glu 318 Glu).

3.2. Comparison of three polymorphisms between RSA patients and control women

Three of the ten detected SNPs with high allele frequencies were chosen for a case–control association study. These SNPs were IVS3+562 del C, IVS 6+61 G→ A and IVS 9+2431 G→ A. The distribution of genotypes and allele frequencies of these three SNPs among RSA patients and the control women are summarized in Table 2. As indicated, no significant differences in genotype distributions and allele frequencies were observed between the two groups. Neither deletion-homozygosity at the IVS3+562 del C site nor AA-homozygosity at the IVS6+61 G→ A polymorphic site was found among RSA and control women.

3.3. Comparison of haplotype frequencies between RSA patients and control women

A total of 6 haplotypes were constructed at the 3 polymorphic sites among RSA and control women (Table 3). Among them, the two most frequent in both study groups were CGA and CGG. Statistical analysis showed no significant difference in their frequency distributions between the two study groups ($P=0.7$). None of the 2 haplotypes were associated with RSA.

Table 2
Distribution of detected SNP genotypes and alleles in RSA patients and the control group using automated sequencing.[a]

INDO genotype and alleles	RSA patients[b] ($N=111$)	Controls[b] ($N=105$)	*P*-value
Genotype			
IVS3+562 del C			
CC	84(77. 8)	86 (81)	
C*[d]	24(22.2)	19 (19)	
**	0 (0)	0 (0)	0.32
Allele			
C	0.88	0.91	
*	0.12	0.9	0.32
Genotype IVS6+61 G→ A			
GG	97(93.3)	95 (94)	
GA	7(6.7)	6 (6)	
AA	0	0	0.52
Allele			
G	0.97	0.97	
A	0.03	0.03	0.66
Genotype IVS9+2431 G→ A			
GG	19(18.6)	25(24.27)	
GA	48(47.1)	54(52.42)	
AA	35(34.3)	24(23.3)	0.17
Allele			
G	0.42	0.51	
A	0.58	0.49	0.13

[a] Values are shown in absolute numbers (percentage).
[b] In certain SNPs the analyzed samples were less than 105 and 111 because of technical problems.
*[d]Deletion.
*Deletion of one nucleotide; **Homozygot (two nucleotide) deletion.

4. Discussion

Indoleamine 2,3-dioxygenase (INDO) is expressed at the maternal–fetal interface and may suppress maternal

Table 3
Comparison of *INDO* haplotype frequency distributions between RSA patients and controls.

No.	IVS3+562delC	IVS6+61 G→ A	IVS9+2431 G→ A	Freq.		*P*-value
				RSA	Controls	
1	C	G	A	0.49	0.44	0.7
2	C	G	G	0.35	0.43	
3	*[a]	G	G	0.07	0.05	
4	*	G	A	0.05	0.05	
5	C	A	A	0.03	NS	
6	C	A	G	NS	0.03	
			Total	99	100	

NS: not seen.
*[a]: deletion.
*Nucleotide deletion.

Table 4
INDO SNP functional consequences using SNPnexsus database.

SNPname	dbSNPs	Gene	Source	Transcripts	Functional consequences (number of transcripts)	Distance to splice
IVS3+562 del C	rs 4259403	INDO	RefSeq	1	Intronic	45
			Ensembl	1	Intronic	45
			VEGA	1	Intronic	45
			UCSC	2	Intronic	45
			AceView	5	Intronic	45
		lervawbu	AceView	1	Intronic	14,749
325 G→ A (Val 109 Ile)	[a]	INDO	RefSeq	1	Coding; NS(V 109 I)	
			Ensembl	1	Coding; NS(V 109 I)	
			VEGA	1	Coding; NS(V 109 I)	
			UCSC	2	Coding; NS(V 109 I)(I), 3UTR(1)	
			AceView	5	Coding; NS(V 109 I)(4), 5UTR(1)	
		lervawbu	AceView	1	Intronic	14,815
IVS6+32 T→ G	[a]	INDO	RefSeq	1	Intronic	31
			Ensembl	1	Intronic	31
			VEGA	1	Intronic	31
			UCSC	2	Intronic	31
			AceView	5	Intronic(4), Coding; NS(V 190 F)(1) 31	
		lervawbu	AceView	1	Intronic	18,661
IVS6+54 T→ A	[a]	INDO	RefSeq	1	Intronic	53
			Ensembl	1	Intronic	53
			VEGA	1	Intronic	53
			UCSC	2	Intronic	53
			AceView	5	Intronic(4), Coding; NS(N 197 I)(I) 53	
		lervawbu	AceView	1	Intronic	18,683
IVS6+61 G→ A	[a]	INDO	RefSeq	1	Intronic	60
			Ensembl	1	Intronic	60
			VEGA	1	Intronic	60
			UCSC	2	Intronic	60
			AceView	5	Intronic(4), Coding; NS(F 197 L)(I)	60
		lervawbu	AceView	1	Intronic	18,690
IVS8+116 T→ G	rs 9298586	INDO	RefSeq	1	Intronic	116
			Ensembl	1	Intronic	116
			VEGA	1	Intronic	116
			UCSC	2	Intronic	116
			AceView	5	3 downstream(3), Intronic(2)	116
		lervawbu	AceView	1	Intronic	20,867
720 C→ T (Asp 240 Asp)	[a]	INDO	RefSeq	1	Coding; S(D240 D)	
			Ensembl	1	Coding; S(D 240 D)	
			VEGA	1	Coding; S(D240 D)	
			UCSC	2	Coding; S(D240 D)(I), 3UTR(1)	
			AceView	4	3 downstream(1), 3UTR(1), Coding; S(D240 D), S(G 93 G)(2)	
		lervawbu	AceView	1	Intronic	21,214
805 G→ A(Val 269 Ile)	a	INDO	RefSeq	1	Coding; NS(V 269 1)	
			Ensembl	1	Coding; NS(V 269 1)	
			VEGA	1	Coding; NS(V 269 1)	
			UCSC	2	Coding; NS(V 269 I)(I), 3UTR(1)	
			AceView	4	3downstream(I), 3UTR, Coding(1); NS(V269I), NS(V22I)(2)	
		lervawbu	AceView	1	Intronic	21,299
IVS9+2431 G→A	rs 3739319	INDO	RefSeq	1	Intronic	28
			Ensembl	1	Intronic	28
			VEGA	1	Intronic	28
			UCSC	2	Intronic	28
			AceView	3	Intronic	28
		lervawbu	AceView	1	Intronic	23871
954 G→ A(Glu 318 Glu)	[a]	INDO	RefSeq	1	Coding; S(E 318 E)	
			Ensembl	1	Coding; S (E 318 E)	
			VEGA	1	Coding; S(E 318 E)	
			UCSC	2	Coding; S(E 318 E)(I), 3UTR (1)	
			AceView	3	3UTR(1), Coding; S, S(E 171 E) (E318 E)(2)	
		lervawbu	AceView	1	Intronic	21,214

NS: non-synonymous; S: synonymous.
[a] Novel SNP.

immune response to the semi-allogeneic fetus (Munn et al., 1998). Recent data demonstrated that CD4+CD25+ regulatory T cells (Treg cells) and the INDO enzyme may cooperate in the induction of maternal tolerance during pregnancy (Saito et al., 2007).

Ligation of CTLA-4, which is expressed on CD4+CD25+ Treg cells, enhances INDO activity in dendritic cells (DC) and macrophages (Fallarino et al., 2003). Miwa et al. (2005) showed that INDO expression in DCs after CTLA-4 treatment is decreased in miscarriages. Therefore, CD4+CD25+ Treg cells and INDO expressing DCs are very important in the maintenance of normal pregnancy. A decreased number and reduced function of Treg cells in women with RSA has been reported (Arruvito et al., 2007). This decrease may affect INDO expression and activity and may result in immunologic pregnancy complications such as RSA. Despite these findings several investigators have reported that INDO functions as facilitator of conversion of naïve T lymphocytes into Treg cells (Fallarino et al., 2006).

In this investigation, we screened exons and exon–intron borders of the INDO gene and identified ten genetic variants in 111 southern Iranian RSA patients. In a similar study Arefayene et al. (2009) identified seventeen genetic variants of INDO in Caucasian and African–American normal subjects in USA. This group also analyzed the functional effect of different variants on INDO enzyme activity.

Three of our identified SNPs had previously been registered in the NCBI SNP database (Table 1). Two of them (rs 9298586 and rs 3739319) were also reported by the Arefayene group (Arefayene et al., 2009). In this study the allele frequency of IVS9+2431 G→A was higher than the Arefayene study (42% VS 1%) which emphasized ethnic differences. There are many other INDO SNPs in the NCBI SNP data base which were not identified in this investigation, because they probably exist in certain regions of the gene that were not sequenced, or alternatively they are lacking in Iranian women. Additional studies are needed to clarify the presence of INDO gene variants in other populations.

For functional annotation of both new and known identified INDO gene SNPs in this study, we used the SNPnexus database. This database is the only tool that provides a comprehensive overview of functional consequences of SNPs on alternatively spliced genes by exploring five different transcriptome and proteome models (Chelala et al., 2008). The results of analysis of the 10 identified SNPs of INDO using the SNPnexus database are summarized in Table 4. Two of five models (UCSC and AceView) utilized by SNPnexus database showed more than one functional consequence, e.g. 3UTR, 5UTR and 3downstream. According to the AceView models INDO variants overlap with isoforms of the lervawbu gene. The distance from splicing sites of intronic SNPs are detailed in Table 4.

To the best of our knowledge and as confirmed by the SNPsnexus database analysis, there is no report in the literature concerning an association between INDO polymorphisms, haplotypes and RSA, therefore we were not able to make a comparative analysis.

In conclusion, according to this study INDO exhibits different genetic variants in different populations and there is no association between INDO polymorphisms and RSA. Other investigations are needed to clarify the genetic variants of INDO in different ethnic groups and the association of its polymorphism with RSA.

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Mutation and Copy Number Analysis in Paroxysmal Kinesigenic Dyskinesia Families

Paroxysmal kinesigenic dyskinesia (PKD [MIM128200]) is a heritable paroxysmal movement disorder characterized by recurrent and brief attacks of involuntary movements.[1,2] Its family histories show an autosomal dominant inheritance pattern. Our previous linkage and haplotype analyses defined the disease locus on 16p11.2-q12.1,[2] Similarly, other linkage studies of PKD assigned the locus to an overlapping region encompassing the centromere of chromosome 16.[3–5] In our previous study, we performed mutation analysis in seven families on 157 genes between D16S3131 and D16S416 (all the genes within this region); however, we failed to identify the causative gene.[1] Based on many linkage studies, we decided to extend the candidate region until more telomeric locus to D16S503 containing 72 RefSeq genes. Because genomic rearrangement could also result in PKD, we also performed copy number analysis for the entire candidate PKD locus.

Here, we describe the results of mutation analysis in 14 PKD families for the 72 genes between D16S416 and D16S503, and the results of copy number analysis in eight PKD families and two sporadic cases.

We collected 14 Japanese families, PKD-1–PKD-14, each of which includes multiple individuals affected by PKD, and two sporadic cases, PKD-S1 and PKD-S2. Among all these families, 64 patients were diagnosed with PKD. Our previous study showed that all affected members in each family have a disease related haplotype on chromosome 16[1,2] except for PKD-1 and PKD-2, which were not analyzed for haplotype because the family members is small.

Direct sequencing of the 72 genes in the segment between D16S416 and D16S503 revealed two substitutions which were not observed among 288 normal controls and not deposited in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) (Table 1). A substitution, g.25190C>T (p.R282C) in *GPR114* found in the family PKD-12, was considered as rare variant because it was not co-segregated with PKD. The remaining one was g.35905C>T in exon 4 of *NLRC5* (NM_032206) resulting in p.T153T, segregated with PKD in family PKD-3. Even though this mutation in *NLRC5* is "silent," it might be a pathogenic because of splicing disturbance.[6] However, a nucleotide g.35905C in *NLRC5* is not so highly conserved in other species, and g.35905C>T would not affect splicing by prediction of NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html) and GENSCAN (http://genes.mit.edu/GENSCAN.html; data not shown).

Copy number analysis using HumanExon510S-Duo BeadChip (Illumina, San Diego, CA) showed a deletion in 16p11.2 (Fig. 1A), but this has already been reported in the Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/). In our previous study, two nonsynonymous substitutions, p.P242T in *SCNN1G* and p.K1063R in *ITGAL*, which were segregated with PKD in one family, were still possible pathogenic mutation for PKD.[1] Structural variants including microdeletions/microduplications within three genes, *ITGAL*, *SCNN1G*, and *NLRC5*, were scanned using array comparative genomic hybridization (aCGH: Agilent Technologies, Santa Clara, CA). Two small deletions not registered in DGV were found within *ITGAL* among several patients (Fig. 1B). However, real-time quantitative PCR revealed genomic alterations in only one PKD patient in the ITGAL region1 and region2 (Fig. 1C). No alteration was found in *SCNN1G* and *NLRC5*. Results of copy number analyses showed no causative copy number changes.

Together with our previous study,[1] we have now analyzed almost all the exons and exon-intron boundaries between

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Table 1. Novel SNPs identified in this study

Gene name	Nucleotide change	Location	AA change	Family	Notes
SLC12A3	g.14369C>T	Exon 11	A464A	PKD-13	Synonymous substitution Observed among 96 controls
NUDT21	g.21841T>C	3′UTR		PKD-11, PKD-14	Observed among 96 controls
PLLP	g.4C>T	5′UTR		PKD-13	Observed among 96 controls
KATNB1	g.8648G>A	Exon 3	S58N	PKD-3, PKD-10, PKD-11, PKD-13, PKD-14	Nonsynonymous substitution Observed among 96 controls
	g.15561G>A	IVS5+6		PKD-14	Near the splice site Observed among 96 controls
	g.16858C>T	IVS8+4		PKD-1, PKD-10, PKD-14	Near the splice site Observed among 96 controls
	g.18427C>T	Exon 12	D409D	PKD-1, PKD-13	Synonymous substitution Observed among 96 controls
	g.19491C>T	Exon 14	P472P	PKD-S2	Synonymous substitution Observed among 96 controls
SNORA46[a]	g.122G>A			PKD-8	Observed among 96 controls
	g.128G>A			PKD-4	Observed among 96 controls
NLRC5	g.35905C>T	Exon 4	T153T	PKD-3	Synonymous substitution Not observed among 288 controls
	g.36018C>T	Exon 4	P191L	PKD-11, PKD-12	Nonsynonymous substitution Observed among 96 controls
SLC38A7	g.4673A>G	Exon 3	T10C	PKD-13	Nonsynonymous substitution Observed among 96 controls
	g.13614G>A	Exon 10	Q378Q	PKD-3	Synonymous substitution Observed among 96 controls
GPR114	g.25190C>T	Exon 9	R282C	PKD-12	Nonsynonymous substitution Not observed among 288 controls

[a]*SNORA46* is a noncoding RNA.IVS, intervening sequence; AA, amino acid.

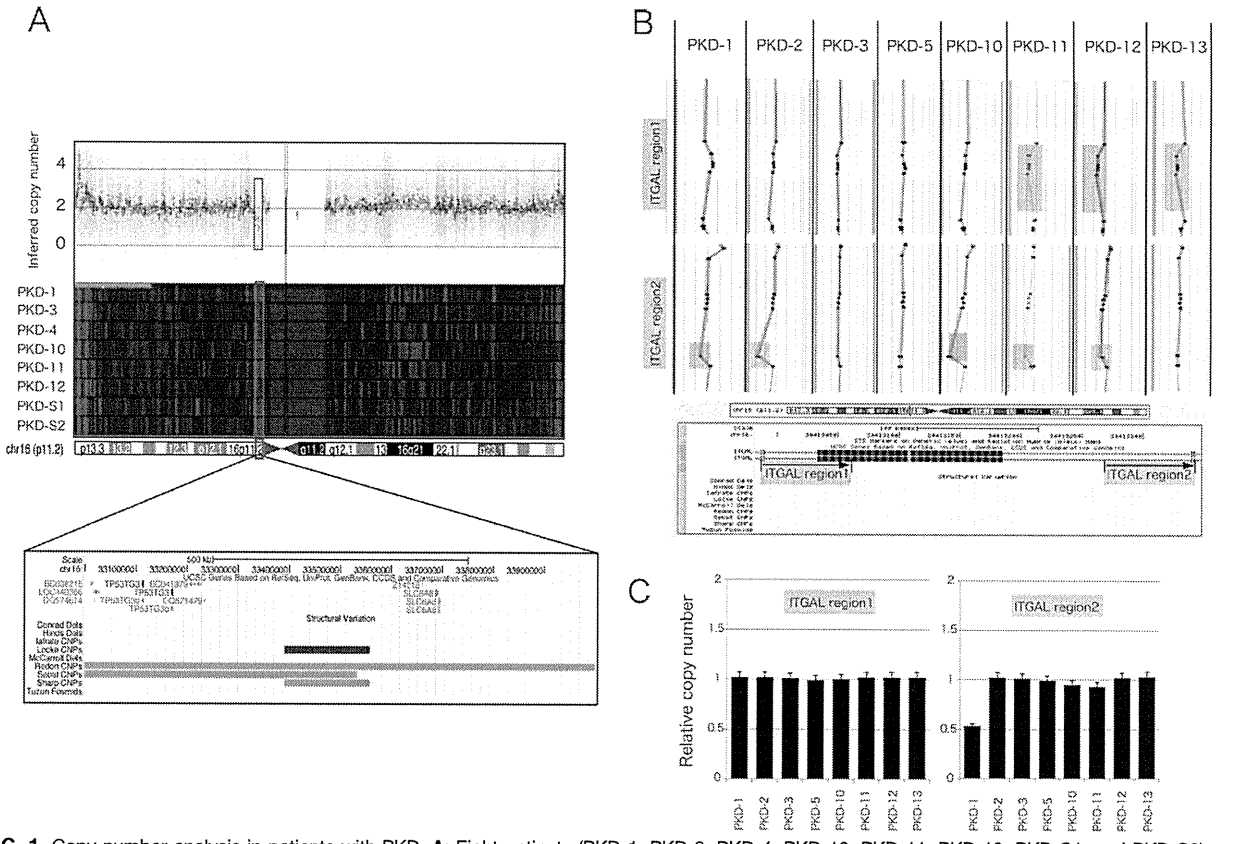

FIG. 1. Copy number analysis in patients with PKD. **A:** Eight patients (PKD-1, PKD-3, PKD-4, PKD-10, PKD-11, PKD-12, PKD-S1, and PKD-S2) are screened for copy number change using HumanExon510S-Duo BeadChip. Representative microarray data for chromosome 16. The region surrounded with a rectangle indicates the deleted region in affected individuals. Three horizontal lines indicate the inferred copy number. The deletion is registered in DGV. **B:** Eight patients (PKD-1, PKD-2, PKD-3, PKD-5, PKD-10, PKD-11, PKD-12, and PKD-13) were screened for copy number change using aCGH. Two microdeleted regions within *ITGAL* detected by array CGH are indicated by gray shaded areas (*ITGAL*, intron 11, Region 1) and light red shaded areas (*ITGAL*, intron 11, Region 2). Array CGH in PKD-11, PKD-12, and PKD-13 showed a ~100 bp loss of copy number in Region 1, and in PKD-1, PKD-2, PKD-10, PKD-11, and PKD-12 showed a ~100 bp loss of copy number in Region 2. **C:** The left and right bar graphs represent the results of quantitative PCR in *ITGAL* Region 1 and Region 2, respectively. No copy number changes were detected in Region 1, and the deletion in Region 2 was detected only in PKD-1, among eight patients with PKD.

D16S3131 and D16S503, but failed to identify the causative gene for PKD. Why have we failed? It is possible that PKD is caused by a recurrent structural aberration beyond the detection level of our experimental approaches. Because the 90th percentile largest gap on the HumanExon510S-Duo BeadChip is 14 kb, it is highly unlikely that our analysis could detect <15 kb structural variants accurately.

The peri-centromeric region (16p11.2–12.1) represents the largest zone of interchromosomal duplications and is composed of ~54 intrachromosomal duplications.[7] It is difficult to find pathogenic copy number changes and base changes in such a complex region. Even though our sequence analysis was comprehensive, some genes could be incomplete. For example, gene conversion in multiple copy number genes would be overlooked in our screening strategy. Furthermore, it is possible that the recurrent or founder intronic mutation cause PKD. den Hollander et al.[8] showed that a recurrent intronic mutation affected the splicing of *CEP290* gene, which resulted in Leber congenital amaurosis. The limitation of our strategy is that most of intronic regions in candidate locus could not be analyzed. We could not have an evidence of founder mutation in Japanese; it is still possible that a founder mutation in an intron is shared in patients.

We conclude that the causative mutation for PKD has not yet been identified. New technologies may be required to identify the PKD mutation in this complex genomic region.

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Imaging Evidence of Nigral Damage in Dystonia Secondary to Disulfiram Intoxication

There are a few reports on patients who developed dystonia or parkinsonism after disulfiram (DSF) intoxication. Such patients had lesions in the pallidostriatal nucleus on brain magnetic resonance imaging (MRI) studies.[1] However, the integrity of the nigrostriatal dopaminergic neurons has not been studied.

We describe a 25-year-old man who developed generalized dystonia following DSF intoxication. [^{18}F]-FP-CIT brain positron emission tomography (PET) studies showed reduced uptake in the bilateral posterior putamen.

A 25-year-old man presented with generalized dystonia following DSF intoxication. At the age of 15, he committed suicide by taking a 15 g of DSF. When he was transported to the emergency room, he was drowsy but had no focal neurological deficits. One month later, he developed action-induced dystonia in both feet. He also developed sudden onset of transient generalized dystonia. The attacks occurred about 10 times a day and lasted up to 5 min. On neurological examinations, he could understand simple questions and his speech and hand writing were unintelligible. He showed initiation delay in the vertical and horizontal saccadic eye movements. When he stretched out his arms, there was no dystonia. His finger tapping was very slow, but there was no fatigue or akinetic block. Once he made fists, he could hardly release them. While tapping his feet, there was dystonia in the toes. His foot tapping was slow bilaterally, and the amplitude was small. There was no motor weakness or sensory deficit. He needed help to stand and walk due to severe dystonic plantar flexion of the feet. There was a mild spasticity in the legs, but no rigidity in the arms and legs. The knee and ankle jerks increased mildly, but plantar reflexes were flexor bilaterally and there was no ankle clonus. Occasionally, he developed sudden onset of transient generalized dystonia, consisting of retrocollis, upward deviation of the eyes, grasp of the hands, and flexion and

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ORIGINAL ARTICLE

Clinical application of fetal sex determination using cell-free fetal DNA in pregnant carriers of X-linked genetic disorders

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As the first step in prenatal diagnosis of X-linked genetic disorders, chorionic villus sampling (CVS) for fetal sex determination is generally performed at 11–13 weeks of gestation. However, as the procedure-related miscarriage rate of CVS is 0.5–1.0%, non-invasive methods such as PCR of cell-free fetal DNA (cff-DNA) in maternal plasma are preferable. Here, we determined fetal sex at 9–12 weeks of gestation using PCR of cff-DNA in three pregnant carriers of Duchenne muscular dystrophy. The fetal sex was accurately determined in all three cases, as confirmed by ultrasound and amniocentesis at 16 weeks (for the two female fetuses) and CVS at 12 weeks (for the one male fetus). This procedure could avoid unnecessary CVS in female fetuses.
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Keywords: cell-free fetal DNA; Duchenne muscular dystrophy; fetal sex determination; prenatal diagnosis; X-linked genetic disorder

INTRODUCTION

As a first step in the prenatal diagnosis of X-linked genetic disorders, chorionic villus sampling (CVS) for fetal sex determination is generally available to pregnant carriers of Duchenne muscular dystrophy (DMD) at 11–13 weeks of gestation. If the fetus is female, she must have a wild-type genotype or a heterozygous DMD mutation, the same as her mother. If the fetus is male, further genetic analysis is necessary, because a male fetus is either wild type or affected by DMD. However, the procedure-related miscarriage rate of CVS is 0.5–1.0%.[1] For CVS, the miscarriage rate was increased in departments performing <136 procedures per year compared with departments performing more than 136 procedures per year, while for amniocentesis the miscarriage rate was only increased in those departments performing <45 procedures per year.[2] In addition, to reach the plateau of learning curve, 400 procedures are needed for CVS, while 30 procedures are needed for amniocentesis.[3,4] CVS is more difficult to master than amniocentesis and the sampling failure rate of CVS is at least three times that of amniocentesis.[2–5] Therefore, to avoid the risks of CVS in pregnant female DMD carriers, a non-invasive procedure for fetal sex determination in early pregnancy is desirable.

In 1997, cell-free fetal DNA (cff-DNA) was found in the maternal plasma, and this discovery offered exciting new avenues for non-invasive prenatal diagnosis.[6] The cff-DNA in maternal plasma is detectable after 7 weeks of gestation and is undetectable 2 h after delivery.[7,8] Fetal DNA in maternal plasma includes placental DNA, and comprises around 3–6% of the total cell-free DNA in maternal plasma during early and late pregnancy, respectively.[8,9] PCR of Y chromosome-specific sequences on cff-DNA from maternal plasma is one such method for non-invasive fetal sex determination until 9 weeks of gestation.[10,11] Compared with CVS, the advantages using cff-DNA in maternal plasma is that the sampling method is non-invasive and carries no risk for the mother or her baby, and that fetal sex can be diagnosed before 11–13 weeks of gestation, which is the best sampling period of CVS. Using this method as the first step, we have outlined a schema for prenatal diagnosis in carriers of DMD as follows (Figure 1): determine fetal sex between 9 and 12 weeks of gestation by nested PCR of cff-DNA in maternal plasma. If the fetus is male, follow-up with CVS at 12 weeks of gestation. If the fetus is female, CVS can be avoided; fetal sex is confirmed later in the pregnancy by ultrasound and amniocentesis at 16 weeks.

Here, we report our experiences regarding the clinical application of using cff-DNA from maternal plasma for the prenatal diagnosis of DMD.

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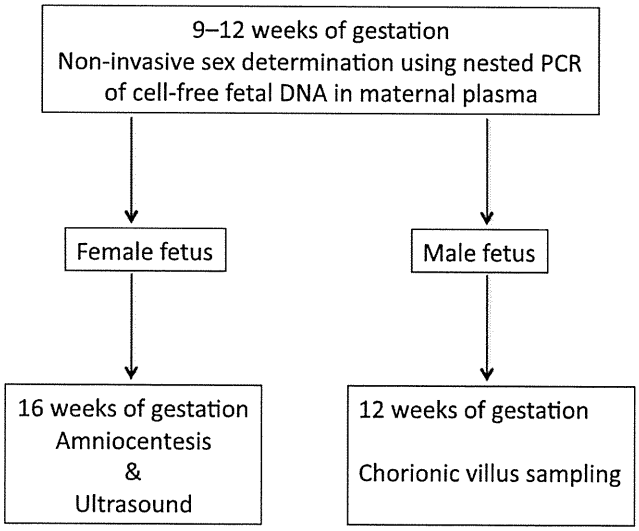

Figure 1 Outline of schema for prenatal diagnosis in pregnant carriers of Duchenne muscular dystrophy.

MATERIALS AND METHODS

All study protocols were approved by the Committee for Ethical Issues on Human Genome and Gene Analysis of Nagasaki University. All the pregnant women and their partners gave written informed consent and received genetic counseling.

Cases

Case 1, a 29-year-old woman, gravida 2, para 2, was referred to our clinic, because she planned to obtain a prenatal diagnosis of DMD at 9 weeks of gestation. Because her first son had DMD, she underwent a genetic test and was diagnosed as a DMD carrier (Figure 2a; subjects II-1 and I-1). During a previous pregnancy, CVS was performed for prenatal diagnosis of DMD at 12 weeks of gestation, and genetic analysis concluded that fetus was male and had no DMD mutation (Figure 2a; subject II-2).

Case 2, a 23-year-old woman, gravida 3, para 1, was referred to our clinic at 7 weeks of gestation. Because her first son had DMD, she underwent a genetic test and was diagnosed as a DMD carrier (Figure 2b; subjects II-1 and I-1).

Case 3, a 29-year-old woman, gravida 3, para 1, was referred to our clinic at 9 weeks of gestation. Because her younger brother had DMD, she underwent a genetic test and was diagnosed as a carrier of DMD, which was caused by deletion of exon 47 in the *DMD* gene (Figure 2c; subjects II-4 and II-2). Her first and third pregnancies were artificial terminations during the early first trimester (Figure 2c; subjects III-1 and III-3). Her first son was diagnosed with DMD, which was caused by the deletion of exon 47 (Figure 2c; subject III-2).

Sampling and extraction of cff-DNA in maternal plasma

Serial EDTA-blood samples (7 ml) were taken from 9 to 12 weeks of gestation. EDTA-blood samples were also taken from 50 women pregnant with a male fetus and 50 women pregnant with a female fetus. The blood samples were centrifuged twice at 3000 *g* to obtain truly cell-free plasma. Cell-free DNA was extracted from 1.6 ml of maternal plasma using a QIAamp blood mini kit (Qiagen, Hilden, Germany) as described previously.[9] DNA was eluted into 30 µl of sterile and DNase-free water.

Sex determination using nested PCR of cff-DNA

Sex determination was performed using a multiplex, nested PCR to amplify the X-specific *FMR1* gene and the Y-specific *SRY* gene. PCR primers and conditions have been described previously.[12] The FMR1-specific sequence and SRY-specific sequence were co-amplified first by two sets of PCR primer pairs, FMR1.1 (5′-CCCTGATGAAGAACTTGTATCTC-3′), FMR1.2 (5′-GAAATTACACACATAGGTGGCACT-3′), SRY1 (5′-CTAGACCGCAGAGGCGCCCAT-3′) and SRY2 (5′-TAGTACCCACGCCTGCTCCGG-3′). PCR reaction was performed in a 20 µl mixture containing 5 µl cff-DNA, 10 pM of each primers, 250 M dNTP, 0.5 U Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 2 µl of 10× PCR buffer (Applied Biosystems), using GeneAmp PCR System 9700 (Applied Biosystems). The nested PCR with primers FMR1.3 (5′-TCGCCTTTCTCAAATTCCAAG-3′), FMR1.2, SRY3 (5′-CATCCAGAGCGTCCCTGGCTT-3′) and SRY4 (5′-CTTTCCACAGCCAC ATTTGTC-3′) was performed in a total volume of 20 µl using 1 µl of the first PCR product as a template. The first and the nested PCR were cycled 40 times at 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. A volume of 10 µl of the nested PCR products were analyzed on 2.0% agarose gels. PCR products of cff-DNA had only the 261-bp fragment from X chromosome—the fetuses were female—while those had both a 261-bp from X chromosome and a 198-bp fragment from Y chromosome and the fetus was, therefore, male. The results of the PCR-based sex determination were confirmed after delivery.

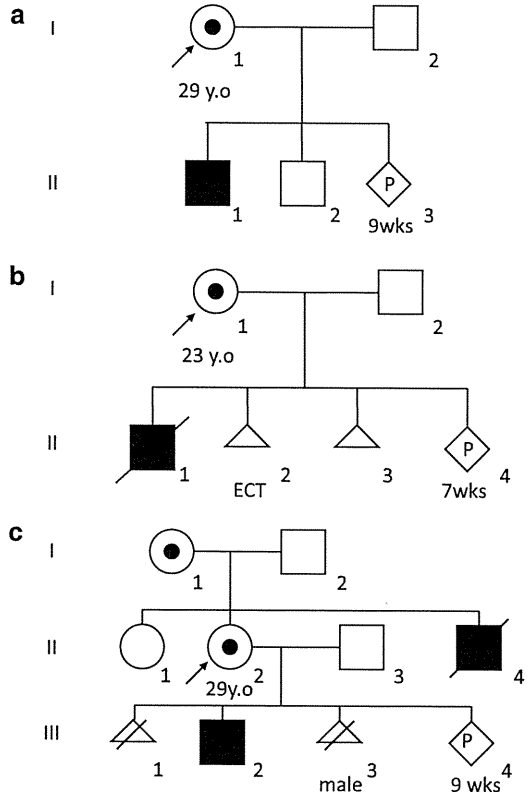

Figure 2 Pedigrees of pregnant carriers of Duchenne muscular dystrophy. (**a**) Case 1; (**b**) Case 2; (**c**) Case 3. ETC indicates ectopic pregnancy. Arrows indicate the cases. Filled squares indicate males affected with Duchenne muscular dystrophy; filled inner circles indicate female carriers. P indicates the current pregnancy.

RESULTS

Accuracy of fetal sex determination using cff-DNA in maternal plasma

Gradient dilution experiments (male DNA was gradually diluted in female DNA background) showed the minimum sensitivity of the nested PCR assay to detect male-specific allele in female DNA background was 1:100 ~ 1:1000 male:female DNA pool (Figure 3). Moreover, fetal gender discrimination was accurate at 9–12 weeks of gestation in 100% of cases. Both X- and Y-specific PCR products were detected in all 50 plasma samples from women pregnant with a male fetus, while only X-specific PCR products were detected in all 50 plasma samples from women pregnant with a female fetus.

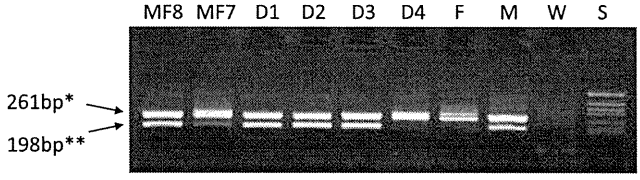

Figure 3 Gradient dilution experiments of the nested PCR assay. Male DNA was gradually diluted in female DNA background. Lanes labeled MF8 and MF7 indicate cff-DNA from a woman pregnant with a male fetus at 8 weeks of gestation and 7 weeks of gestation, respectively; D1, D2, D3 and D4 indicate 1:1, 1:10, 1:100 and 1:1000 male:female DNA pool, respectively; F and M indicate female and male DNA, respectively; W, distilled water; S, size marker (pIBI31/*Msp* I). * and ** show a 261 bp PCR product from X chromosome and a 198 bp PCR product from Y chromosome, respectively.

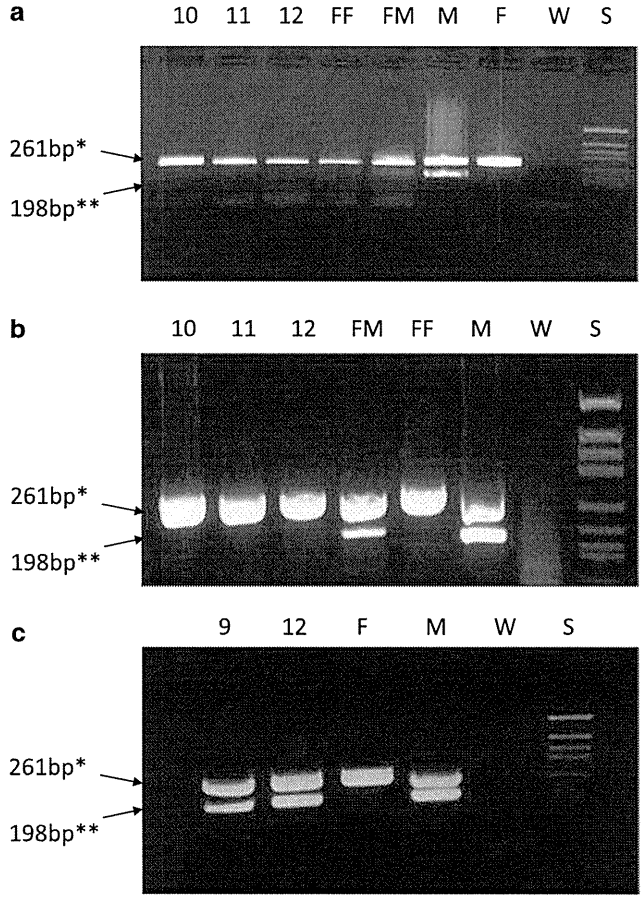

Figure 4 Fetal sex determination using PCR of cell-free fetal DNA (cff-DNA) in maternal plasma. (**a**) Case 1; (**b**) Case 2; (**c**) Case 3. Lanes labeled 9, 10, 11 and 12 indicate cff-DNA at the respective week of gestation; FF and MF indicate cff-DNA from a woman pregnant with a female or male fetus, respectively; F and M indicate female and male DNA, respectively; W, distilled water; S, size marker (pIBI31/*Msp* I). * and ** show a 261 bp PCR product from X chromosome and a 198 bp PCR product from Y chromosome, respectively.

Prenatal fetal sex determination using maternal plasma samples

For sex determination of the fetuses of the above three Cases, nested PCR of cff-DNA in maternal plasma was performed at 9–12 weeks of gestation. PCR products from female DNA showed only a 261-bp fragment, while those from male DNA had both a 261-bp and a 198-bp fragment (Figures 4a–c). PCR products of cff-DNA from Cases 1 and 2 had only the 261-bp fragment—the fetuses were female (Figures 4a and b)—while those from Case 3 had both fragments (Figure 4c) and the fetus was, therefore, male. According to our protocol, ultrasound examination and amniocentesis were provided to Cases 1 and 2 at 16 weeks of gestation: both fetuses showed normal female genitalia and a normal female karyotype (46, XX). In Case 3, CVS was performed at 12 weeks of gestation and the fetal sex was confirmed as male. Exon 47 of *DMD* was detected in DNA from a cultured villus sample and from his parents, but not in DNA from his affected elder brother (data not shown).

DISCUSSION

Compared with the conventional techniques of prenatal diagnosis, including CVS and amniocentesis, the main advantage using cff-DNA in maternal plasma is that the sampling method is non-invasive and carries no risk for the mother or her baby. Another advantage is its reliability in determining fetal sex very early, from 7 weeks of gestation.[10,11] Amniocentesis is only available after 15 weeks of gestation, because before then it carries a risk of talipes equinovarus. Similarly, CVS is only performed between 11 and 13 weeks of gestation, because before 10 weeks it is possibly related to an increased risk of limb reduction defects.[13,14] Early prenatal diagnosis is desirable because, in the case of a positive diagnosis, termination of pregnancy is more stressful during the second trimester than during the first. Although CVS is a first trimester option for the prenatal diagnosis of X-linked genetic disorders,[1] early non-invasive fetal sex determination could avoid unnecessary CVS of female fetuses, and also give more time for parents to consider the invasive procedure in the case of male fetuses. This technique could be applied to the detection or exclusion of genetic sequences that are not present in mother, that is, fetal rhesus D status in D-negative mothers, or genetic conditions inherited from the father or arising *de novo*.[15,16]

False positive and false negative results in sex determination using cff-DNA are still a concern. False positives can result from technical issues such as contamination, or clinical abnormalities such as the presence of a non-identical vanishing twin or a confined placental mosaicism/chimerism.[9,16] False negative results due to failure to detect the Y-chromosome sequence may still be problematic. To reduce these problems, maternal plasma analysis can be paired with ultrasound examination and amniocentesis; ultrasonic identification of fetal sex is possible within the first 12 weeks of gestation.[17] Even if ultrasonography reveals a misdiagnosis, prenatal diagnosis is still feasible through amniocentesis. Sample processing protocols clearly affect the quality of cell-free DNA in the maternal plasma. When only a single centrifugation step is performed, cff-DNA is detected in non-pregnant women, reflecting contamination by residual circulating cells from previous pregnancies.[18,19] A second microcentrifugation step is essential to obtain truly cell-free plasma.[20] To find the best procedure for non-invasive sex determination using cff-DNA, the use of multi-copy sequence as target sequence on Y chromosome is one of the option, because the assay using multicopy *DYS 14* sequence demonstrated a 10-fold better detection rate and quantification limits for fetal DNA compared with the assay using single copy *SRY* gene.[21]

In conclusion, fetal sex determination using cff-DNA in maternal plasma could avoid unnecessary CVS of female fetuses, decreasing parental anxiety, the cost and the attached risk. As such, genetic counselors and obstetricians should be aware of the potential for earlier and safer prenatal diagnosis, although it should be stressed that the technique is still in development and has potential to be used more widely for the purposes of family balancing or for preference of a particular sex.[2,23] Because of the ethical, social and legal issues

surrounding fetal sex determination,[22,23] we recommend that the clinical use of this procedure should be reserved for those fetuses at risk of a gender-specific inherited disorder.

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ORIGINAL ARTICLE

Epidemiology of human papillomavirus genotypes in pregnant Japanese women

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To investigate the pre-vaccination epidemiology of genital human papillomavirus (HPV) infections and genotypes in pregnant Japanese women, we performed Pap smear tests and HPV genotype testing in patients attending Nagasaki University Hospital and collaborating hospitals from August 2007 to July 2010. Serial uterine cervical specimens were obtained from 151 pregnant women. The HPV test was positive on the first visit in 54 women (35.8%; 54/151, average age 30). A total of 49 women (32.5%; 49/151) were infected by at least one high-risk HPV and 5 women were infected by only low-risk HPV. The three most prevalent high-risk HPV genotypes were HPV 52 (31.5%; 17/54), HPV 16 (29.6%; 16/51) and HPV 31 (13.0%; 7/51). The HPV infection pattern (negative, single infection and multiple infection) differed significantly according to the pregnancy trimester (χ^2-test; $P<0.01$(Pearson)). Among HPV-infected pregnant Japanese women, HPV52 was the most common genotype. The second most common genotype was HPV16, and these two genotypes accounted for ~60% of HPV-positive pregnant women. Infection with multiple HPV genotypes was observed more frequently in the first trimester of pregnancy and the pattern of infection changed significantly depending on pregnancy stage.
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Keywords: epidemiology; genotype; HPV; infection; pregnancy; uterine cervical neoplasia

INTRODUCTION

Persistent infections with human papillomavirus (HPV) are recognized as a major cause of cervical cancer. Genital infections with HPV are very common, and these infections are transmitted by sexual contact.[1] In most cases, HPV infections disappear naturally in a relatively short time period and carry only a small risk of disease development;[2–4] however, some investigators believe that pregnancy affects the host immune system; for example, pregnancy is believed to reduce seroreactivity against HPV infection.[5] Estrogen and progesterone have been shown to activate the upstream regulatory region of HPV18 (see ref. 6). Thereby altering HPV clearance rates compared with non-pregnant women. During persistent infection, important factors of disease susceptibility include HPV genotypes and viral characteristics, such as the distribution of each type in the population and the evasive ability against the host's immune system. It is not clear how persistent infection relates to the host, such as how the host immune system reacts against a specific HPV genotype and how sexual behavior in pregnant women affects infection rates. In particular, there are very few data detailing HPV genotype prevalence in pregnant Japanese women. To determine the distribution of HPV infections in pregnant Japanese women, we performed HPV genotype testing.

MATERIALS AND METHODS

Study population and sample collection

Cytology and HPV DNA test samples were collected by six hospitals that collaborated with Nagasaki University Hospital from August 2007 to July 2010. All pregnant women whose first visit during pregnancy was to one of our collaborating hospitals were invited to join the study. An obstetrics doctor explained the study aim, procedures and complications. We did not inform participants of the HPV genotyping results. Exclusion criteria were patients who had previously received therapeutic surgery or who had histologically confirmed non-squamous neoplasms. We registered 151 pregnant women having a routine Pap test screen for the study. Specimens were taken with a Cervex brush (Rovers Medical Devices, Oss, Netherlands) and suspended in 10 ml of SurePath preservative fluid (Becton Dickinson, Franklin Lakes, NJ, USA). We used sample from the same vial for cytology and for HPV genotype testing. Cervical specimens for HPV genotyping

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were taken at each visit from participants who received regular follow-up examinations.

The study protocol was approved by the ethical review board of Nagasaki University and of the other hospitals involved. All women were informed of the purpose of the study and gave their consent.

HPV genotyping test

Genotyping of HPV DNA in the SurePath preservative fluid was performed using the Linear Array HPV Genotyping Test kit (Roche Diagnostics, Indianapolis, IN, USA). The kit uses PGMY09/PGMY11 primers[7] to amplify the L1 conserved region. Following PCR amplification, hybridization of the HPV amplicon was performed using an array of oligonucleotide probes that allowed independent identification of individual HPV genotypes. This kit can detect 37 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108 (89)). For consistency with previous studies, we considered 16 HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) as high-risk genotypes that are related to cervical cancer.[8–10]

RESULTS

A total of 151 women were enrolled in the study. In all, 79 women were in the first trimester of their pregnancy, 18 were in the second trimester, 51 were in the third trimester and 3 were postpartum. Fifty-four women (35.8%; 54/151), with an average age of 30, were positive for HPV on their first hospital visit. Forty-nine women (32.5%; 49/151) were infected by at least one high-risk HPV and five women were infected by only low-risk HPV. The three most prevalent high-risk HPV genotypes were HPV 52 (31.5%; 17/54), HPV 16 (29.6%; 16/51) and HPV 31 (13.0%; 7/51) (Table 1). The HPV infection pattern (negative, single infection and multiple infection) was significantly different depending on the trimester of the pregnancy (χ^2-test; $P<0.01$ (Pearson)).

Table 1 Prevalence of HPV infection in pregnant Japanese women

	Trimester			
	1st	*2nd*	*3rd*	*Total (%)*[a]
n	79	18	51	148
HPV negative	48	7	39	94
Single infection	17	9	10	36 (66.7)
Multiple infection	14	2	2	18 (33.3)
HPV type				
(high-risk type)				
16	7	6	3	16 (29.6)
18	2	2	1	5 (9.3)
31	5	0	2	7 (13.0)
33	3	0	0	3 (5.6)
51	2	0	0	2 (3.7)
52	14	2	1	17 (31.5)
53	1	0	0	1 (1.9)
56	3	0	0	3 (5.6)
58	2	1	2	5 (9.3)
68	2	0	0	2 (3.7)
82	1	0	1	2 (3.7)
(Low-risk type)				
6	2	1	0	3 (5.6)
39	1	0	1	2 (3.7)
42	0	0	1	1 (1.9)
54	1	1	0	2 (3.7)
61	3	0	1	4 (7.4)
71	0	0	1	1 (1.9)
84	0	0	2	2 (3.7)
CP6108 (89)	2	0	0	2 (3.7)

Abbreviation: HPV, human papillomavirus.
[a]The denominator was the number of HPV-positive women.

DISCUSSION

The influence of pregnancy on the natural course of HPV infection is not understood. Several reports show that the prevalence of HPV infection in pregnant women is variable at 10–60%, depending on age, region and HPV detection methods.[11–14] The HPV infection rate in pregnant women in this report was 35%. There are few reports of HPV infection rates in pregnant Japanese women. One report by Takakuwa *et al.*[15] showed the prevalence of HPV in pregnant Japanese women to be 12.5%. The difference between this report and our results may be because of the detection method used, PCR-reverse hybridization (our study) and PCR-restriction fragment length polymorphism (Takakuwa *et al.*), and the number of detectable HPV genotypes.

Several other studies[16,17] show the clearance of HPV infection may accelerate in the third trimester and postpartum. In our study, the HPV infection pattern differed significantly according to the pregnancy trimester. In particular, in the first trimester, multiple HPV infection was observed more often than in the later periods. This observation may be explained by changes in sexual behavior and/or by immunological factors. Pregnant women tend to be less sexually active and their immune response against HPV is reduced. The accelerated clearance of HPV may be due to the host immune system normalizing during the third trimester.[18]

Our results showed that HPV 16 and HPV 52 were the two most common genotypes among pregnant Japanese women. Our data among 154 non-pregnant Japanese women also showed that HPV 16 and HPV 52 were the two most common genotype. The prevalence of HPV genotype in Japanese pregnant women may not show pregnant-specific features. Other reports using HPV genotyping tests also showed that HPV 52 was a more common genotype among Japanese individuals who had either normal cytology or cervical neoplastic lesions compared with individuals in other countries.[10,19,20]

In Japan, a commercial cervical cancer vaccine finally became available after December 2009. This study has some limitations because we obtained data from pregnant women; however, our data from pre-vaccination women on the distribution of genital HPV infections in the region where HPV 52 is more prevalent are important for the understanding of the cross-reactivity of a bivalent HPV vaccine.[17,21]

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Identification of Novel Schizophrenia Loci by Homozygosity Mapping Using DNA Microarray Analysis

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Abstract

The recent development of high-resolution DNA microarrays, in which hundreds of thousands of single nucleotide polymorphisms (SNPs) are genotyped, enables the rapid identification of susceptibility genes for complex diseases. Clusters of these SNPs may show runs of homozygosity (ROHs) that can be analyzed for association with disease. An analysis of patients whose parents were first cousins enables the search for autozygous segments in their offspring. Here, using the Affymetrix® Genome-Wide Human SNP Array 5.0 to determine ROHs, we genotyped 9 individuals with schizophrenia (SCZ) whose parents were first cousins. We identified overlapping ROHs on chromosomes 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 19, 20, and 21 in at least 3 individuals. Only the locus on chromosome 5 has been reported previously. The ROHs on chromosome 5q23.3–q31.1 include the candidate genes histidine triad nucleotide binding protein 1 (*HINT1*) and acyl-CoA synthetase long-chain family member 6 (*ACSL6*). Other overlapping ROHs may contain novel rare recessive variants that affect SCZ specifically in our samples, given the highly heterozygous nature of SCZ. Analysis of patients whose parents are first cousins may provide new insights for the genetic analysis of psychiatric diseases.

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Introduction

Schizophrenia (SCZ) is categorized as a severe chronic debilitating psychosis that affects approximately 1% of the global population. Although genetic factors are reported to contribute to the disease and multiple responsible loci have been identified from linkage analysis and case-control association studies, there have been few reproducible results to date [1].

Morrow et al. (2008) [2] suggested that homozygosity mapping is a powerful tool not only for investigating single gene defects but also for rare genomic variants in complex traits. They observed homozygous deletions in patients with autistic disorders and concluded that genomic alterations might be a subset of disease-causing mutations in chromosomal regions. The increased susceptibility to SCZ observed in consanguineous families suggests that genomic recessive variations may be involved in its etiology. [3–5] Considering this and other results, we hypothesized that homozygosity mapping, including identical by descent (IBD) analysis, would be a highly constructive method for identifying the loci responsible for SCZ.

We hypothesized that runs of homozygosity (ROHs) could contribute to SCZ by a recessive effect. We use the term "ROH" [6] instead of loss of heterozygosity (LOH) for regions where homozygous genotypes are contiguous because LOH implies heterozygous deletions or hemizygosity, while ROH suggests consecutive homozygous regions. Recessive effects are obtained by genetic variations including single nucleotide variations, small insertions/deletions, structural variations, and chromosomal rearrangements. These variations may affect amino acid sequences or the control of gene expression, including small RNA expression.

Here, we describe a homozygosity mapping strategy that consisted of 2 stages (Figure 1). The first stage aimed to find the appropriate size threshold for autosomal ROHs that would distinguish ROHs specifically existing in the offspring of first-cousin marriages from those that commonly exist in the offspring of non-consanguineous marriages. By comparing the size distribution of ROHs between the offspring of first-cousin marriages and non-consanguineous marriages, we concluded that ROHs >2.1 Mb in size in the offspring of consanguineous marriages can be assumed to be IBD segments from an individual 3 generations before. The second stage aimed to find shared ROHs among patient with SCZ using 2 models. In Model I, an autosomal ROH size threshold was applied to filter out smaller ROHs. Larger ROHs were assessed to find overlaps among the patients. In Model II, after filtering by the ROH size threshold, ROHs shared by the siblings of patients and ROHs of other patients were assessed to find overlaps. The overlapping ROHs we identified potentially contain SCZ causative regions that are specific to our samples because of the heterogeneous nature of SCZ.

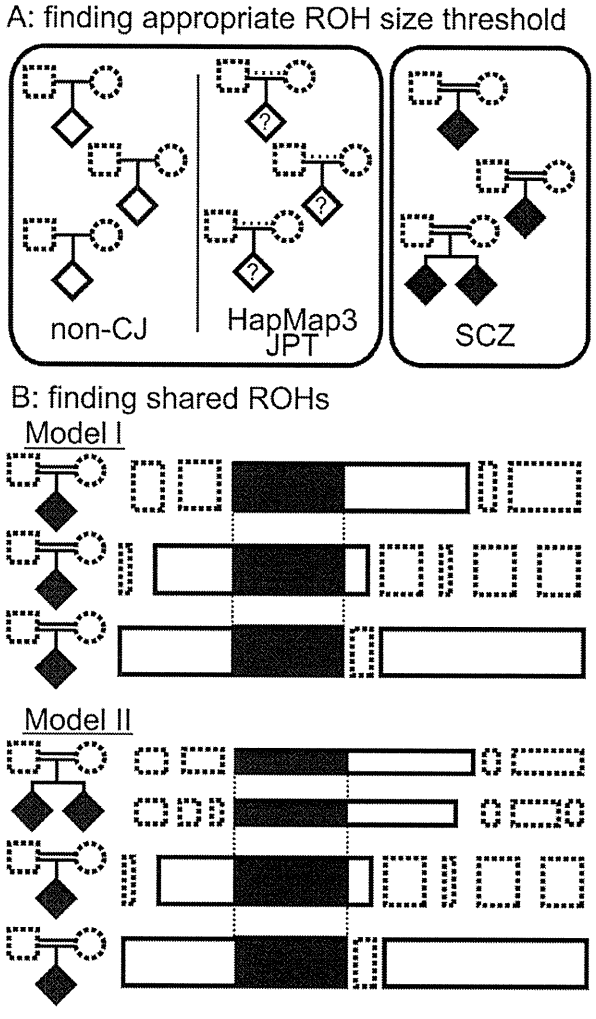

Figure 1. Two-stage design of this study. A, the first stage was to find an appropriate autosomal run of homozygosity (ROH) size threshold to distinguish specific ROHs from the offspring of first-cousin marriages from ROHs in the offspring of non-consanguineous marriages. The size distribution of ROHs in our non-consanguineous Japanese (non-CJ) and schizophrenia (SCZ) samples was compared. Non-CJ samples are the offspring of non-consanguineous marriages that were validated by interview. Here, SCZ samples were used as the offspring of first-cousin marriages regardless of phenotype. Samples from parents were not used in this study (dashed squares and circles). To confirm our strategy, we also assessed HapMap3 JPT samples, which do not have information for phenotypes or family consanguinity (dashed and solid lines between parents). B, the second stage was to find shared ROHs among the SCZ samples as patients with schizophrenia. In Model I, an autosomal ROH size threshold was applied to filter out smaller ROHs (dashed open boxes). Larger ROHs (solid open boxes) were assessed to find overlaps among patients (solid boxes). In Model II, after filtering by the ROH size threshold, ROHs shared by the siblings of patients and ROHs of other patients were assessed to find overlaps. In this study, the gender of the samples was not matched (diamonds) because we only evaluated autosomal ROHs.
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Materials and Methods

1. Samples

A total of 9 subjects with SCZ (3 males and 6 females, aged 31–56 years) (SCZ individuals) were recruited to this study after being diagnosed as having typical paranoid schizophrenia by a certified psychiatrist (N.K.) using the *Diagnostic and Statistical Manual of Mental Disorders*, Fourth Edition, Text Revision (DSM-IV-TR) and the *Structured Clinical Interview for DSM-IV Axis I Disorders* (SCID). The study received ethics approval from the Committee for Ethical Issues on Human Genome and Gene Analysis at Nagasaki University, Japan. All of the patients were from the main islands of Japan, excluding Okinawa. We obtained written informed consent from all participants. The consanguineous patients were from 8 first-cousin marriages. Seven individuals (patients a to g) were unrelated and 2 were siblings (patients h-1 and h-2). We also recruited 92 healthy individuals from non-consanguineous marriages (non-CJ individuals) from the main islands of Japan, excluding Okinawa. We confirmed consanguinity by interview. We did not match for gender in the SCZ and non-CJ individuals because we only intended to analyze autosomal chromosomes.

After obtaining written informed consent, genomic DNA was isolated from peripheral blood. We did not collect blood samples from the patients' parents, except for 1 patient, or siblings; however, we confirmed that they had no history of psychiatric illness, with the exception of the older brother of patient g, by direct interview or from the medical records of the other related individuals.

Furthermore, we also assessed the International HapMap Project [7] phase 3 data of the Japanese in Tokyo (HapMap3 JPT) to evaluate the non-CJ individuals. Raw signal intensity files (CEL files) obtained using Affymetrix Genome-Wide Human SNP Array 6.0 (Affy6.0) were downloaded from http://www.hapmap.org/.

2. Microarray analysis

We performed genome-wide SNP genotyping of 9 SCZ samples and 92 non-CJ samples using the Affymetrix Genome-Wide Human SNP Array 5.0 (Affy5.0) according to the manufacturer's instructions. Our microarray data is MIAME compliant and the raw data has been deposited in the CIBEX database (CIBEX accession number: CBX141).

3. ROH detection

We generated the CHP genotype files from the CEL signal intensity files using the BRLMM-P genotype calling program [8,9]. For the detection of ROHs, we analyzed the CHP files with a hidden Markov model (HMM)-based ROH detection function of the Partek® Genomics Suite (Partek GS) software version 6.5 build 6.11.0207 (Partek, St. Louis, MO, USA). We applied the following default HMM parameters: max probability = 0.99, genomic decay = 0 (disabled), genotype error = 0.01, and default frequency = 0.3. We did not adopt the baseline files.

Detected ROHs were statistically analyzed and visualized (Figures 2 and 3; Tables 1 and 2) by using in-house scripts written in the R language [10]. The optimization of histogram bandwidths and the estimation of the probability density distributions were performed using the "KernSmooth" package of R [11].

Furthermore, to validate the data quality of our non-CJ samples, we also compared our data to HapMap3 JPT. Affy6.0 raw signal intensity data in CEL files were subjected to allele calling using Birdseed software version 2 [12]. SNP genotypes of shared loci between Affy6.0 and Affy5.0 were extracted and processed as well as the non-CJ and SCZ datasets to detect ROHs.

4. Detection of potential genetic loci for SCZ by overlapping ROHs

To detect the overlapping ROHs among the SCZ dataset, the identified ROHs were filtered by a size threshold on Partek GS,

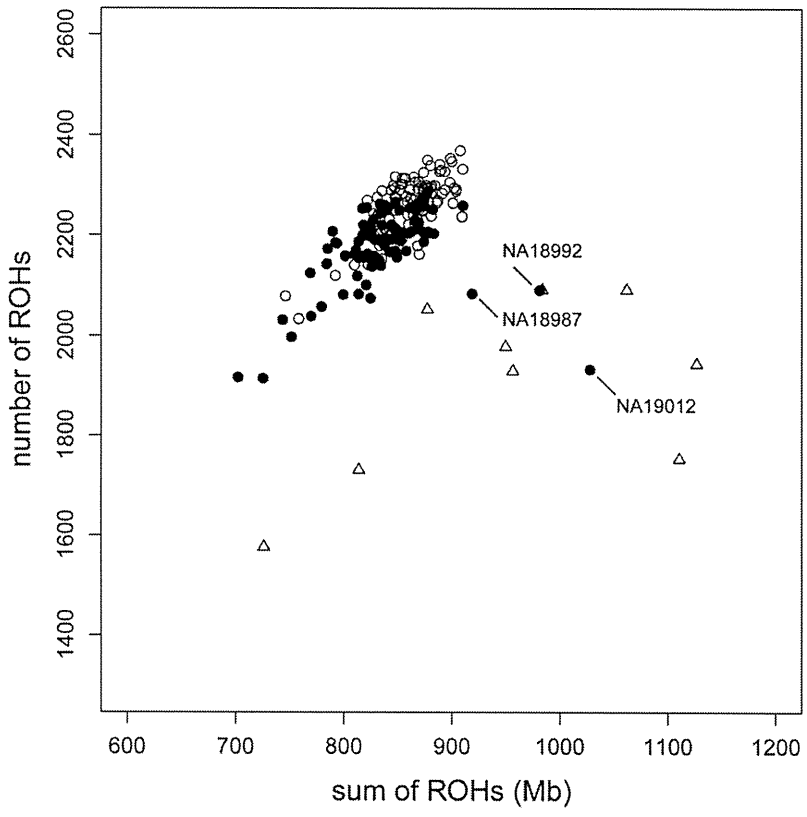

Figure 2. Distribution of the size and number of individual autosomal runs of homozygosity (ROHs). Sums and total numbers of individual ROHs are shown by circles and triangles indicating unrelated Japanese individuals (non-CJ: 92 samples) and the offspring of first-cousin marriages with schizophrenia (SCZ: 9 samples), respectively.
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analyzed using an in-house Ruby script (available on request) to generate a table of overlapping ROHs, and visualized with Partek GS. Then, we extracted the loci shared among more than 3 unrelated individuals (Model I) (Table S1). Furthermore, on the basis of the hypothesis that concordant sibling cases share causal loci, we detected the loci shared among 2 sibling cases (h-1 and h-2) (Model II) and found the ROHs that were shared by 1 or more of the unrelated samples (Table 3).

Results

1. Determination of the ROH size threshold discriminating the offspring from non-consanguineous and first-cousin marriages

We genotyped 440 794 SNPs in each individual. Genotype calling rates for each sample ranged from 97.23–98.83% and their call rates were high and accurate enough for their subsequent evaluation. We utilized the data from 92 non-CJ and 91 HapMap3 JPT samples in addition to the data from 9 SCZ individuals.

Our homozygosity mapping strategy utilized differences in the length distribution of ROHs between offspring from consanguineous and non-consanguineous marriages. Individuals from consanguineous families are expected to have an increased number of longer ROHs containing autozygous segments. These segments were also expected to be discriminated by their length from ROHs containing homozygous segments by chance or by linkage disequilibrium (LD). To demonstrate the strategy, we performed detailed comparisons of the length distribution of ROHs between the non-CJ, HapMap3 JPT, and SCZ datasets.

We initially plotted the total number and size of ROHs in the non-CJ, HapMap3 JPT, and SCZ datasets (Figure 2). The non-CJ and HapMap3 JPT datasets clustered together, except for 3 individuals in HapMap JPT. These 3 outlier individuals, NA18987, NA18992 [13], and NA19012 [14], have been assumed to be from consanguineous families; indeed, the distribution of these samples was similar to that of our offspring from first-cousin marriages (Figure 2).

We then analyzed the length distribution of ROHs in the non-CJ and SCZ datasets. Bar plot histograms of the length of ROHs were obtained and the probability density curves were estimated by the "KernSmooth" package in R (Figure 3A–D). Descriptive statistics of these plots are also shown in Table 1. Both datasets produced bell curve-like distributions in the $\log_{10}$ scale on the x-axis to indicate the length of each ROH; however, the SCZ dataset showed a secondary peak in the larger ROH region. We expected that the autozygous region from the founders of the third ancestral generation (great-grandparents) would be larger in the SCZ dataset than in the non-CJ dataset, in whom LD may encompass ROHs by chance. The proportion of larger ROHs in the SCZ dataset was clearly higher than in the non-CJ dataset. As we can expect that 1/16 of the whole genome in the offspring of first-cousin marriages would be autozygous regions from their great-grandparents, we highlighted the graphs in Figure 3B and

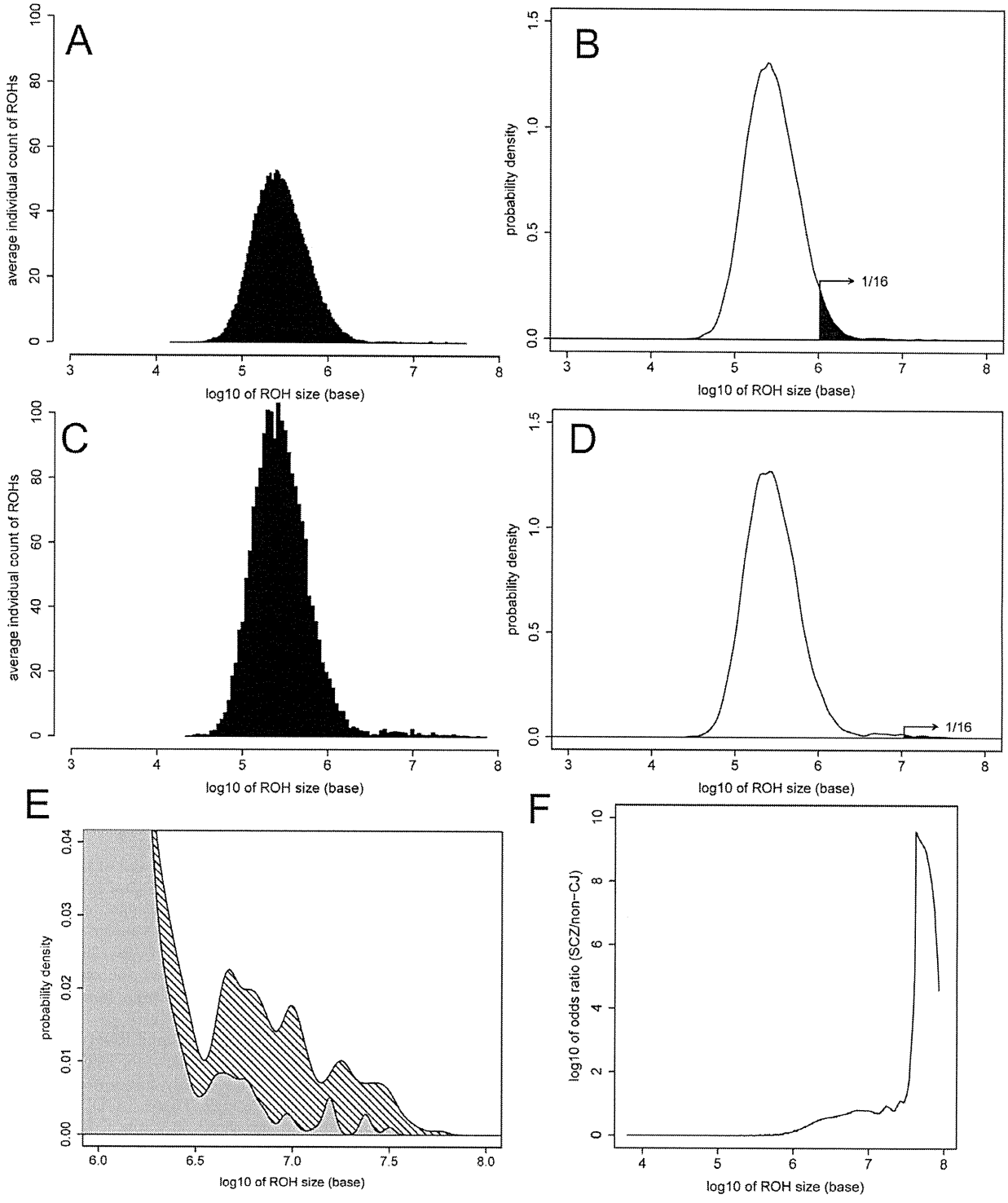

Figure 3. Size distribution of autosomal runs of homozygosity (ROHs). In the size distribution plot of non-consanguineous Japanese (non-CJ; A and B) and schizophrenia (SCZ; C and D) samples, the x-axis indicates the ROH size (log_{10} scale). A and C, individual average frequency of the ROHs as histograms. B and D, estimated probability density corresponding to each histogram. Black areas shows 1/16 (6.25%) of autosomes, which is equivalent to the expected sum of autozygous regions in the offspring of a first-cousin marriage. E, enlarged overlap of B (gray) and D (hatched). F, SCZ/non-CJ odds ratio plot. X-axis indicates the size of the ROHs (log_{10} scale). Y-axis (log_{10} scale) indicates the ratio of areas exceeding the given ROH size threshold in the estimated probability distributions of the SCZ and non-CJ datasets.
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Table 1. Autosomal runs of homozygosity (ROHs) size distribution, where descriptive statistics of ROH sizes were detected with Partek GS.

Dataset	N	Minimum[a]	Mode[b]	Maximum[c]	Average sum[d]
HM3JPT[e]	88	19 750 (14)	256 499 (27)	32 000 000 (1252)	831 159 144
non-CJ[f]	92	18 160 (14)	248 288 (27)	32 250 000 (1921)	859 784 793
SCZ[g]	9	27 380 (14)	258 488 (38)	57 810 000 (9896)	956 266 858

[a]Minimum ROH size in all individuals from each dataset.
[b]Mode ROH size in all individuals from each dataset.
[c]Maximum ROH size in all individuals from each dataset.
[d]Average sum is the average total ROH size per individual from each dataset.
[e]The International HapMap Project phase 3 Japanese in Tokyo. Three samples, NA18987, NA18992, and NA19012, of 91 samples are omitted because they are potentially the offspring of a consanguineous marriage.
[f]Non-consanguineous Japanese.
[g]Schizophrenia.
Numbers are in bases, and the numbers in parentheses are the included probe sets.
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3D at the point where the total sum of length in the upper tail of the ROH distribution reaches 179.2 Mb, which is 1/16 of the 2 867 732 772 bases total size of the autosomal haploid genome, according to the statistics from the NCBI Build 36.1 assembly (2006) [16]. This analysis suggested that it is highly probable that the longer ROHs would be inherited from the great-grandparents; however, it should be mentioned that genomic regions with less recombination tend to have longer ROHs.

To show further differences in the probability density distribution of the SCZ and non-CJ individuals, we also plotted an SCZ/non-CJ odds ratio (OR) plot (Figure 3F and Table 2), which indicates the ratio of probability for the existence of ROHs in each dataset over a given threshold length. To determine the overlapping ROH regions shared among the SCZ dataset, we adopted OR = 3.0 and the corresponding threshold of 2 137 962 bases to ensure practical power and to detect smaller IBD regions by recombination.

2. Determination of potential SCZ genetic loci by overlapping ROHs

The sum lengths of the overlapping regions among 0–7 independent family patients are shown in Figure 4, and the calculated percentage sum length among a given number of patients and more in the autosomal genome were as follows: 100%, 51.7%, 13.6%, 6.0%, 1.9%, 1.3%, and 0.6%. Considering the statistics, we adopted a minimum of 3 patients for identifying candidate loci. Figure 5 shows a schema of the overlapping ROHs within autosomes and their positions are summarized in Table S1.

Overlapping ROHs found in 3 or more SCZ individuals on chromosomes 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 19, 20, and 21 (Figure 5A) suggested that many loci are potentially associated with SCZ in our patients. Only the locus on chromosome 5 has been reported in a previous linkage analysis of SCZ [16]. The ROHs were expanded by the analysis of 4 additional individuals; however, no additional loci were detected (data not shown). The locus on chromosome 5q23.3–q31.1 included the regions containing the histidine triad nucleotide binding protein 1 (*HINT1*) and acyl-CoA synthetase long-chain family member 6 (*ACSL6*) genes. Our results suggest that recessive variants of these candidate genes could be involved in the pathogenesis of SCZ in our patients.

In the analysis of 2 siblings (h-1 and h-2) from a first-cousin marriage, we searched for the ROH regions shared by the siblings as a single gene defect. The detection of loci shared by the siblings and 1 or more unrelated individuals demonstrated ROHs on chromosomes 1, 5, 7, 8, 10, 12, 13, 16, 17, 19, and 21 that might be causative for SCZ (Figure 5B). Those loci did not include any previously reported candidate genes. Interestingly, among the loci detected in Figure 5A and 5B, there were no overlapping loci identified in this study.

Table 2. Thresholds, individual average sums of runs of homozygosity (ROHs), its ratio in the autosomal genome, and the individual average encompassed number of ROHs corresponding to the odds ratios.

Odds ratio	Threshold (base)	Non-CJ[a] dataset			SCZ[b] dataset		
		sum (base)	Autosomal ratio (%)	# of ROHs	sum (base)	Autosomal ratio (%)	# of ROHs
1.3	1 000 000	185 411 092	6.5	93.2	420 200 807	14.7	123.6
2.000	1 548 817	110 468 918	3.9	30.6	341 258 405	11.9	52.7
3.0	2 137 962	81 383 855	2.8	13.8	309 296 125	10.8	33.8
4.0	3 630 781	65 633 075	2.3	7.6	288 028 919	10.0	25.4
5.0	5 128 614	53 423 627	1.9	4.7	263 695 116	9.2	19.8
10.0	24 547 089	7 925 263	0.3	0.3	85 167 811	3.0	2.7

[a]Non-consanguineous Japanese.
[b]Schizophrenia.
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