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.

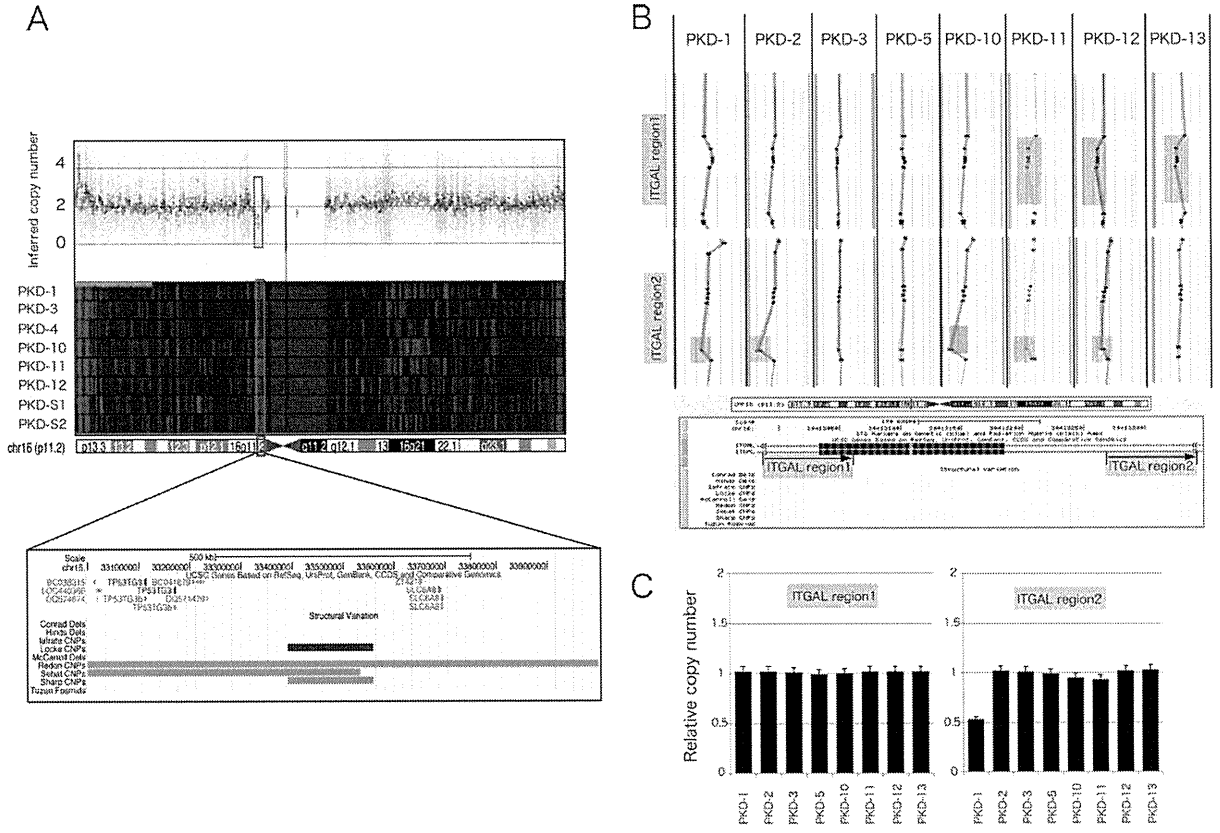

FIG. 1. Copy number analysis in patients with PKD. **A:** Eight patients (PKD-1, PKD-3, PKD-4, PKD-10, PKD-11, PKD-12, PKD-S1, and PKD-S2) are screened for copy number change using HumanExon510S-Duo BeadChip. Representative microarray data for chromosome 16. The region surrounded with a rectangle indicates 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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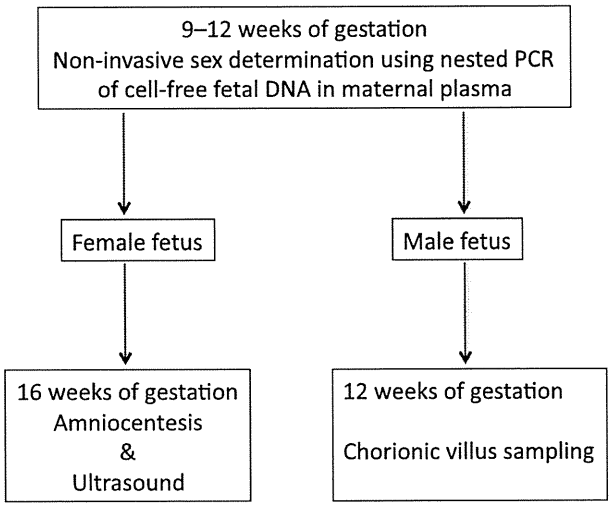

Figure 1 Outline of schema for prenatal diagnosis in pregnant carriers of Duchenne muscular dystrophy.

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.

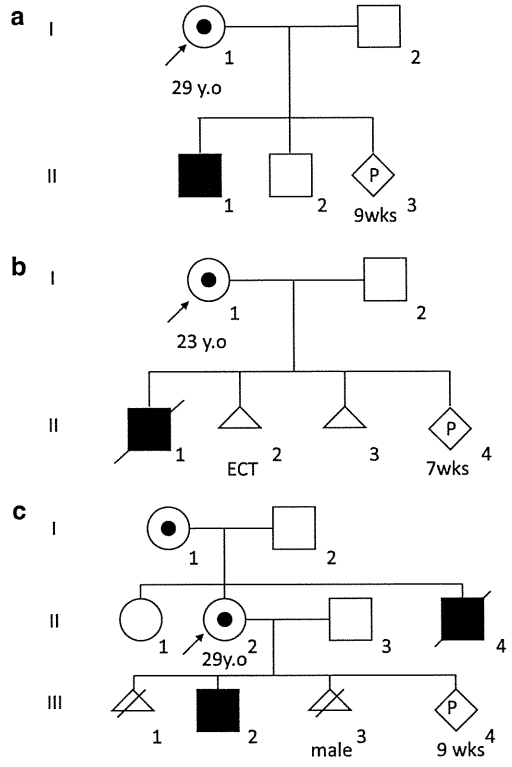

Figure 2 Pedigrees of pregnant carriers of Duchenne muscular dystrophy. (**a**) Case 1; (**b**) Case 2; (**c**) Case 3. ETC indicates ectopic pregnancy. Arrows indicate the cases. Filled squares indicate males affected with Duchenne muscular dystrophy; filled inner circles indicate female carriers. P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

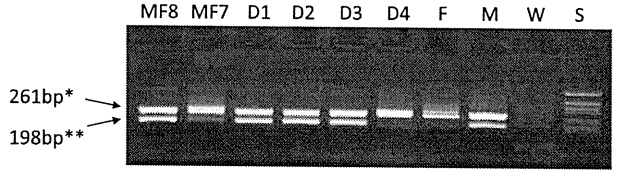

Figure 3 Gradient dilution experiments of the nested PCR assay. Male DNA was gradually diluted in female DNA background. Lanes labeled MF8 and MF7 indicate cff-DNA from a woman pregnant with a male fetus at 8 weeks of gestation and 7 weeks of gestation, respectively; D1, D2, D3 and D4 indicate 1:1, 1:10, 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.

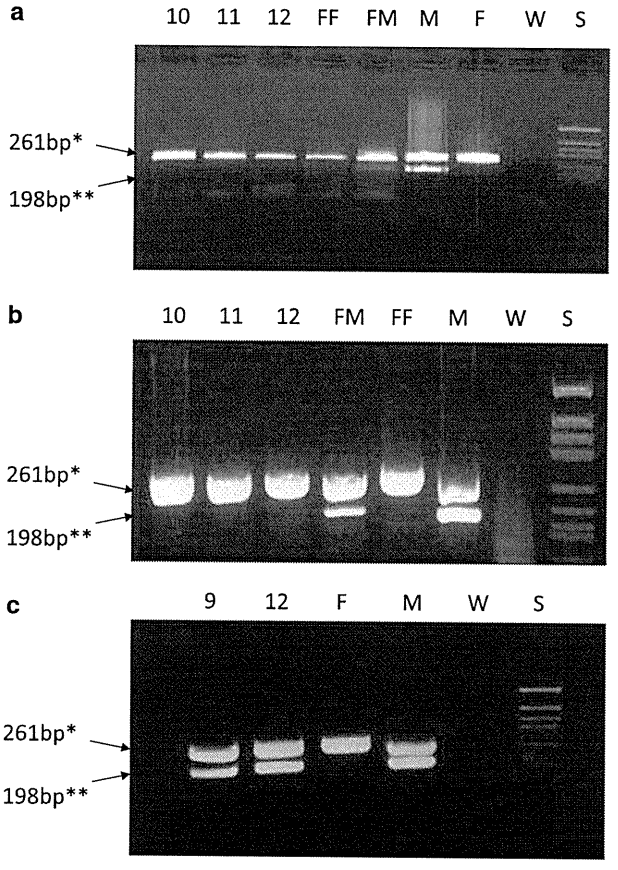

Figure 4 Fetal sex determination using PCR of cell-free fetal DNA (cff-DNA) in maternal plasma. (**a**) Case 1; (**b**) Case 2; (**c**) Case 3. Lanes labeled 9, 10, 11 and 12 indicate cff-DNA at the respective week of gestation; FF and MF indicate cff-DNA from a woman pregnant with a female or male fetus, 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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PLoS one

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.

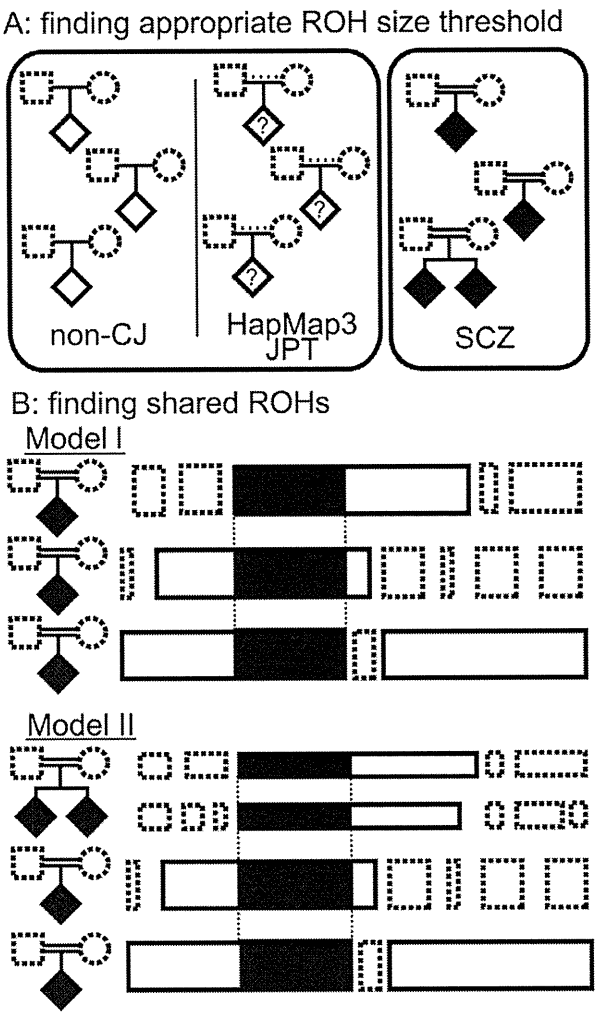

Figure 1. Two-stage design of this study. A, the first stage was to find an appropriate autosomal run of homozygosity (ROH) size threshold to distinguish specific ROHs from the offspring of first-cousin marriages from ROHs in the offspring of non-consanguineous marriages. The size distribution of ROHs 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,

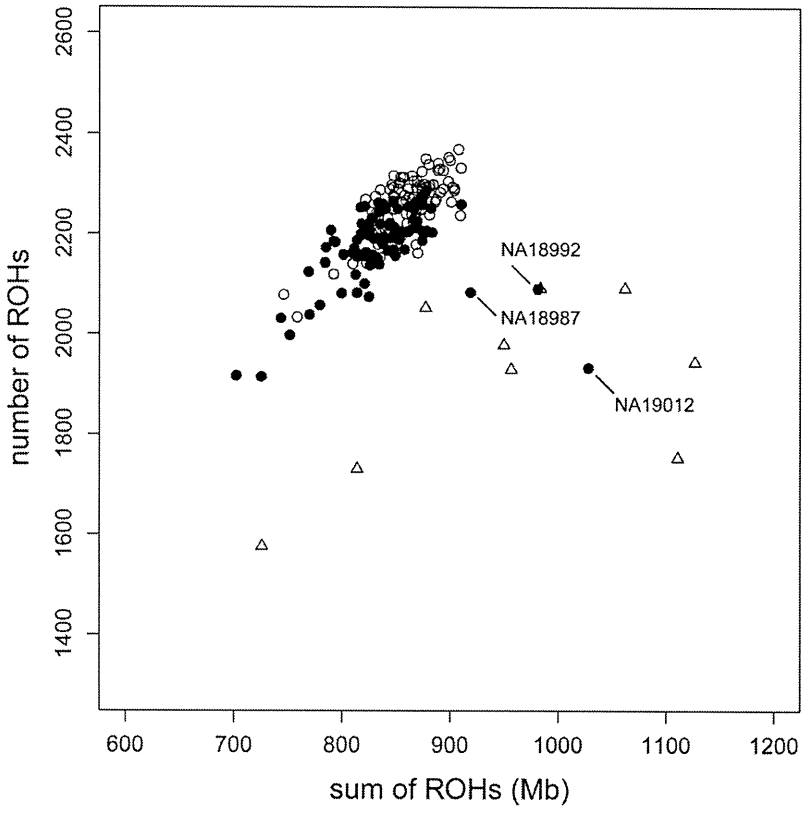

Figure 2. Distribution of the size and number of individual autosomal runs of homozygosity (ROHs). Sums and total numbers of individual ROHs are shown by circles and triangles indicating unrelated Japanese individuals (non-CJ: 92 samples) and the offspring of first-cousin marriages with schizophrenia (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

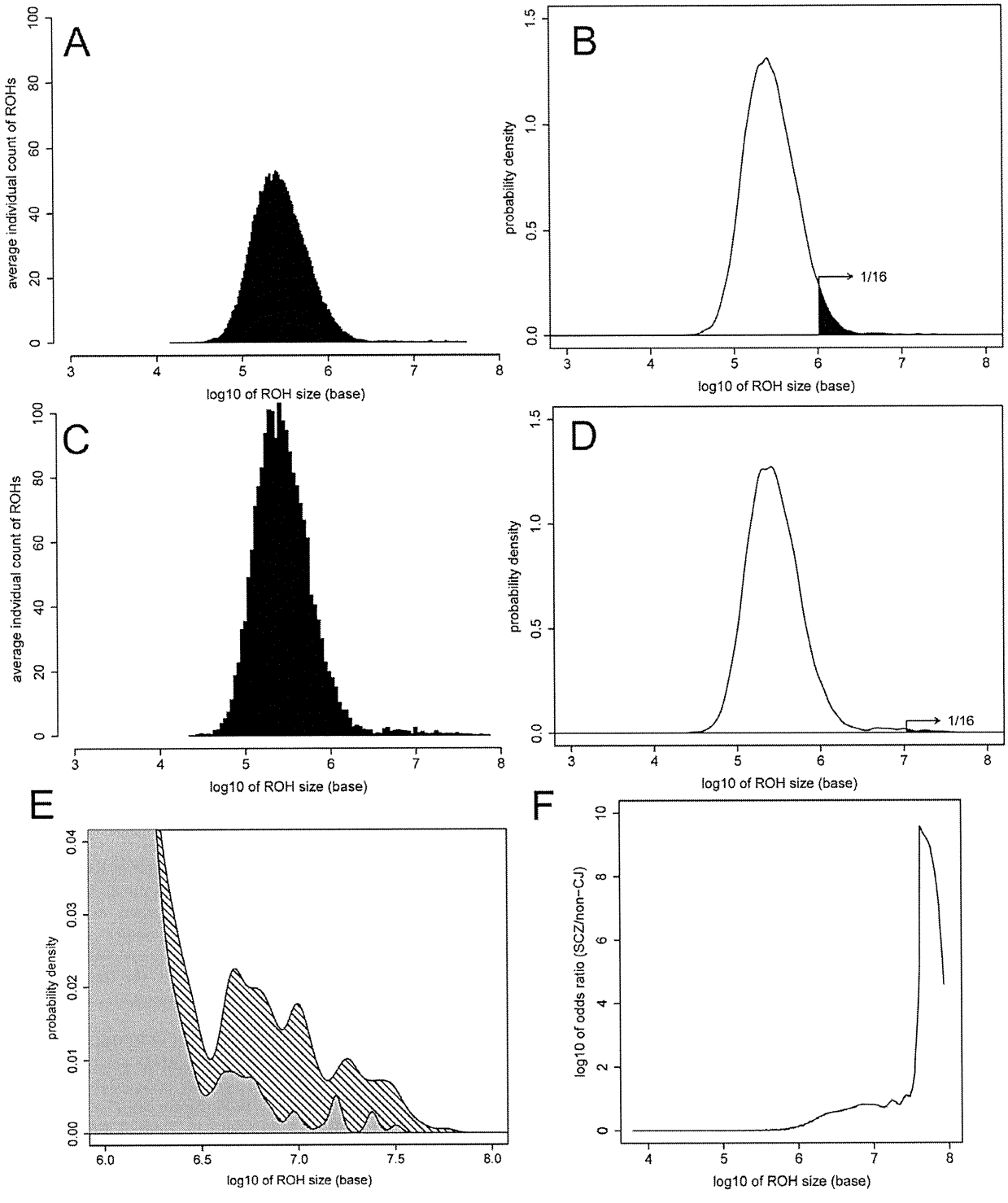

Figure 3. Size distribution of autosomal runs of homozygosity (ROHs). In the size distribution plot of non-consanguineous Japanese (non-CJ; A and B) and schizophrenia (SCZ; C and D) samples, the x-axis indicates the ROH size (log_{10} scale). A and C, individual average frequency of the ROHs as histograms. 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.
doi:10.1371/journal.pone.0020589.t001

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
2.0 00	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.
doi:10.1371/journal.pone.0020589.t002

Table 3. Novel loci identified in this study that are different from those in Table S1, for the segments overlapping in more than 1 unrelated individual and the common regions between the 2 siblings (cases h-1 and h-2).

Chromosome	Start	End	Samples	# Samples[a]	Length	Cytoband
1	146258078	148749860	h-1, h-2, a	3	2491783	1q21.1-q21.2
5	45437574	49631829	h-1, h-2, d	3	4194256	5p12-q11.1
5	117360252	120214932	h-1, h-2, f	3	2854681	5q23.1
5	120214932	122586267	h-1, h-2, f, g	4	2371336	5q23.1-23.2
7	57594442	62282881	h-1, h-2, b, f	4	4688440	7p11.2-q11.21
8	129121122	131617749	h-1, h-2, b	3	2496628	8q24.21-q24.22
8	132434559	139244531	h-1, h-2, b	3	6809973	8q24.22-24.23
10	37363792	37599485	h-1, h-2, e	3	235694	10p11.21
10	37599485	37874740	h-1, h-2, e, g	4	275256	10p11.21
10	37874740	42217616	h-1, h-2, c, e, g	5	4342877	10p11.21-q11.21
12	33982292	36255461	h-1, h-2, a, d	4	2273170	12p11.1-q11
13	35366458	43580724	h-1, h-2, g	3	8214267	13q13.3-14.11
16	28924029	29606107	h-1, h-2, c	3	682079	16p11.2
16	29606107	29657036	h-1, h-2, c, f	4	50930	16p11.2
16	29657036	29680943	h-1, h-2, c, d, f	5	23908	16p11.2
16	29680943	31277953	h-1, h-2, b, c, d, f	6	1597011	16p11.2
16	34467305	34647935	h-1, h-2, a, b, c, d, f, g	8	180631	16p11.1
16	34647935	45122807	h-1, h-2, a, c, d, f, g	7	10474873	16p11.1-q11.2
16	45122807	47094922	h-1, h-2, a, b, c, d, f, g	8	1972116	16q11.2-q12.1
17	29659797	32811528	h-1, h-2, a	3	3151732	17q12
19	37676724	40349191	h-1, h-2, a	3	2672468	19q13.11-13.12
21	19821557	20188026	h-1, h-2, g	3	366470	21q21.2

[a]Number of individuals (including h-1 and h-2) who shared the region; for example, 5 indicates that 3 other individuals shared the common region of the 2 siblings.
doi:10.1371/journal.pone.0020589.t003

Discussion

1. Samples

We recruited 9 offspring from first-cousin marriages (SCZ) and 92 from non-consanguineous marriages (non-CJ). As shown in Figure 2, our non-CJ dataset and publicly available HapMap3 JPT datasets showed a common cluster, except for the presence of 3 outliers that have been reported to be potentially from consanguineous families [14,15]. This concordance suggests that our experimental quality and data processing approaches were appropriate. In this study, we analyzed a limited number of samples; however, homozygosity mapping was a reasonable strategy to adopt because it requires relatively smaller number of samples than case-control studies. We did not use samples from the parents of patients in this study because these are not very informative in our strategy. On the other hand, affected and unaffected siblings in single families are strongly informative in homozygosity mapping, and we are continuously recruiting additional siblings for future study.

2. ROH analysis

Most of the previous homozygosity mapping studies were based on genotypes derived from microsatellites or simple tandem-repeat polymorphisms (STRP). The highly polymorphic nature of multi-allelic STRP markers is suitable to cover the whole genome with a fewer numbers of markers. However, recent DNA microarray technologies have enabled massive genome-wide SNP genotyping to be performed in a short time. The problem with homozygosity mapping based on SNPs is the accurate detection of regions with ROHs. As SNPs have a less informative biallelic nature, using the naïve definition of an ROH as just a contiguous homozygous region may skew the detection of ROHs because of the frequency of low minor allele SNPs, genotyping errors, and "no-call" SNPs.

The solution to this problem using the Affymetrix Human Genotyping 500K arrays and Illumina Infinium HumanHap300v2 arrays was the application of ROH detection bins sliding through each chromosome to filter out low SNP density bins and to allow the small number of heterozygous SNPs and no-call SNPs to be placed in a bin [6,17]. An alternative method to detect ROHs is to adopt an HMM. Partek GS software implements the HMM-based "LOH detection" algorithm. A similar algorithm is also implemented in the Affymetrix GeneChip Chromosome Copy Number Analysis Tool (CNAT), as described in the CNAT user guide [18]. The HMM-based algorithm of these tools takes not only the information of adjacent SNP genotypes but also the heterozygosity of SNPs as a reference baseline calculated from the genotyping results in the reference samples or the *a priori* default frequency. This method is expected to more accurately detect ROH regions that reflect actual recombination.

Selection of the reference population for the baseline data is crucial for the HMM-based detection of ROHs. If the reference population is carefully selected to match the background of the case population, the baseline generated from the observation of actual SNPs in the reference population can omit ROHs resulting from LD and regions with low SNP density, such as centromeres. However, if strict matching of the used population background is difficult, use of the fixed default heterozygous frequency, whose

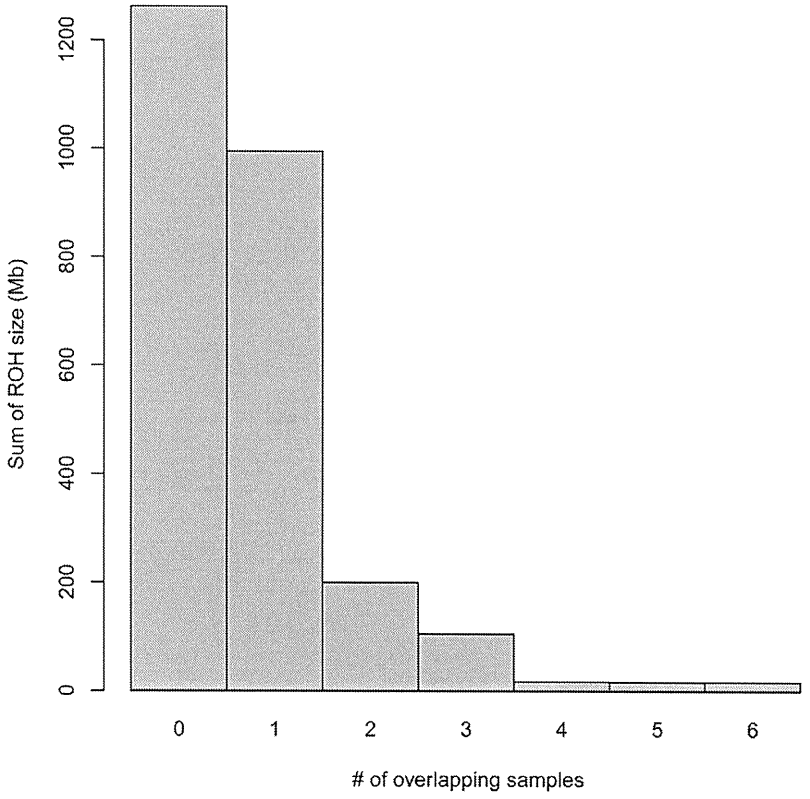

Figure 4. Sum of run of homozygosity (ROH) lengths and number of overlapping patients, excluding patient siblings. Y-axis indicates the sum of ROH lengths shared by a given number of patients. The zero column indicates the sum of ROHs not shared by any of the samples.
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default value is 0.3, still has the advantage of minimizing false-negatives in the detection of ROHs.

To determine the optimal length threshold of ROHs to extract autozygous segments from whole ROHs, we adopted OR = 3 for the analysis. This approach may work well when a large enough reference sample is available. When a reference population is not available, a threshold where the sum of the ROH length in the upper tail of its distribution is equal to the theoretical autozygous length of a genome, that is, 1/16 of a genome in the offspring of a first-cousin marriage, could be another option. In our SCZ dataset, the threshold using this approach was approximately 10.6 Mb.

Our results demonstrated obvious differences in the proportion of the length distribution of ROHs between the non-CJ and SCZ datasets. A recent report on European populations, including endogamy subpopulations, has shown that a higher proportion of individuals in endogamy subpopulations have ROHs longer than 1.5 Mb compared with other subpopulations [6]. Our scatter plot of the individual total number and size of ROHs (Figure 2) is not fully in agreement with this previous report, although the non-CJ dataset made a cluster and showed a positive correlation (Pearson product-moment correlation coefficient r = 0.773), and the SCZ dataset was scattered and showed a weak positive correlation (r = 0.432). This may be explained by the fact that the previous report excluded ROHs <500 kb to ignore ROHs that potentially resulted from LD and removed hemizygous deletions of ROHs. In this study, we did not adopt a strategy to filter ROHs by their size before the analyses because the discrimination of autozygous regions and LD simply by size is essentially impossible. Adopting a baseline file derived from a strictly matched population in the HMM-based detection of ROHs can be used instead. Additionally, differences in genotyping platforms with different SNP densities may affect the size distribution of ROHs. Although our data from the sparser Affymetrix Genotyping 10k SNP panel produced a similar bell curve-like ROH size distribution, the whole curve was shifted to the right (data not shown).

The size distribution of ROHs for a given population is affected by its inbreeding coefficient (F). Studies of consanguineous marriages in subpopulations from Japan during the 1980s compared the F values for Japan (F = 0.00134) to those in Kuwait (F = 0.0219), India (F = 0.02313), England (F = 0.00017), and the United States (F = 0.00003) [19,20]. These reports have also shown that despite the decrease in consanguineous marriages in Japan, local subpopulations have higher F-values. The same tendency has also been shown by a genealogical study that estimated inbreeding rates in large and semi-isolated populations on the basis of historical changes in population size [21]. Recently, the importance of studying endogamous populations has been stressed [22]; however, populations with intermediate F-values have advantages for our homozygosity mapping approach. This approach uses the differences in the size distribution of ROHs in a case population consisting of offspring from consanguineous marriages and a control population consisting of offspring from non-consanguineous marriages. A high F-value population may not have clear distribution differences between cases and controls. On the other hand, finding a sufficient number of cases in low F-value populations may not be easy. From this standpoint, an intermediate F-value population, such as the Japanese population,


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Figure 5. Overlapping autosomal runs of homozygosity. Each autosome is shown horizontally with the number of overlapping samples (upper) and chromosome ideograms (lower). Centromeres are shown by hatched boxes. A, overlapping segments shared among 1 (isolated) to 7 samples in a total of 9 patient samples. B, overlapping segments shared by 2 patient siblings (h-I and h-II) and an additional 1–4 patient samples.
doi:10.1371/journal.pone.0020589.g005

represents an interesting dataset for our homozygosity mapping approach, as was shown previously in the Costa Rican population [23,24].

We presented here some threshold lengths of ROHs to detect IBD regions from great-grandparents. As recombination will, of course, occur everywhere by chance, small autozygous regions could be overlooked with the threshold shown here. However, no systematic analyses have so far identified IBD regions in consanguineous marriages by whole-genome SNP typing. Our method shown here, which 1) detects longer ROHs in each individual and 2) aligns ROHs and identifies overlapping regions, will be helpful for autosomal recessive disorders and also for complex disorders resulting from rare variants. If collecting patients in geographically and historically isolated areas is possible, this homozygosity mapping approach is likely to be successful. Nonetheless, the effectiveness of homozygosity mapping for complex disorders remains controversial [4]. We believe that we can uncover new candidate loci through the application of whole-genome SNP typing to homozygosity mapping because of its high density genomic coverage and high-throughput ability.

3. Possible novel loci for schizophrenia

We identified several putative SCZ loci that are presented in Figure 5 and Tables S1 and 3. In our study, we assumed the 2 models outlined in the Methods section. Model I was designed to find shared causal loci among unrelated individuals. For the other model, we hypothesized that the siblings shared the same causal loci; thus, Model II was designed to find the common loci between the siblings and unrelated individuals.

For Model I (Table S1), from the analysis of 7 unrelated individuals, the loci included the 5q23.3–q31.1 region that was previously identified by linkage analysis in the Irish population [16]. Among the genes that mapped to 5q23.3–q31.1, *HINT1* [25,26] and *ACSL6* [27] were previously reported to be possibly associated with SCZ. In patients from consanguineous families we analyzed, homozygous genomic variations may be causative for the disease (Figure 5 and Tables S1 and 3). However, small sample size in our study may be a limiting factor to generalize such conclusions. In common diseases such as psychiatric disorders, including SCZ and bipolar disorders, especially in familial cases or in cases from relatively isolated areas, rare variants possibly contribute more than common variants to the disease phenotype [28–30].

On the basis of the rare variant-common disease hypothesis, it is appropriate that the genetic etiology between sibling cases and other unrelated cases may be various. In addition, our results suggested that multiple loci influenced the susceptibility to SCZ, as other reports have suggested [31].

We presented here the systematic analyses of the homozygosity mapping method using whole genome SNP typing, and we identified ROHs that potentially contain SCZ causative recessive regions that are shared among our samples. When we explain SCZ as a result of the homozygous state of rare variant mutations, the number of overlapping individuals may be challenging, as it is possible that each individual has a different variation. The heterogeneity of SCZ may explain the lack of overlap for our results with previously reported regions [32,33]; moreover, our methodology has a limitation for detecting causative genes that are included in shorter ROHs by chance. We have shown that the Affymetrix Genome-Wide Human SNP Array 5.0 or 6.0 could be applied to special cases including first-cousin marriages to identify genomic variations. Increasing number of samples obtained from patients from consanguineous families with SCZ is important to make our results more meaningful. Furthermore, we plan to analyze genetic variants in updated ROHs by the next-generation sequencing technologies.

Supporting Information

Table S1 Novel loci identified in this study.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: NK KI-Y HO. Performed the experiments: NK ST HM. Analyzed the data: SO AI TK NN KT. Contributed reagents/materials/analysis tools: SO AI TK NN KT. Wrote the paper: NK KI-Y HO.

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The Breast Journal

LETTER TO THE EDITOR

Intracystic Papillary Carcinoma of Breast Harbors Significant Genomic Alteration Compared with Intracystic Papilloma: Genome-wide Copy Number and LOH Analysis Using High-Density Single-Nucleotide Polymorphism Microarrays

To the Editor:

Intracystic papillary breast tumors (ICPT) consist of benign papillomas, carcinomas in situ, and carcinomas with invasion, and they account for approximately 10% of benign breast tumors and less than 1% of malignant tumors, respectively (1,2). In breast lesions, indication for surgery is usually determined by pathological diagnosis together with radiologic findings, but differential, preoperative diagnosis of papillary carcinoma from papilloma is very difficult, even following needle biopsy (3) because of their nonspecific radiologic characteristics and their modest cytological and histologic appearance (4). To avoid excessive surgical intervention, another diagnostic procedure needs to be developed.

Cytogenetic studies of breast papillary tumors are limited, and cytogenetic differences between papillomas and papillary carcinomas are still controversial. Tsuda et al. (5,6) reported that papillary carcinomas have frequent changes in gene copy number and loss of heterozygosity (LOH), whereas papillomas did not show any gene copy number alteration or LOH at 16q and 1q. Boecker et al. (7) also reported that conventional comparative genomic hybridization (CGH) did not reveal any gene copy number change in papillomas. On the other hand, Lininger et al. (8) and Cristofano et al. (2) demonstrated that LOH at 16p or 16q was frequent in both papillomas and papillary carcinomas.

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The purpose of this study was to determine the profile of genomic alterations in breast ICPT and to explore the possibility of using high-density oligonucleotide SNP arrays as the basis of a novel diagnostic method of ICPT. Ten formalin-fixed paraffin-embedded (FFPE) breast ICPT were obtained from the Department of Pathology, Nagasaki University Hospital. The samples included five benign papillomas (Pap), three papillary carcinomas in situ (PurePC), and two papillary carcinomas with invasion (PCinv). Pathological diagnosis was independently determined by two pathologists. Clinicopathological findings of these tumors are provided in Fig. 1 and Table 1.

Extracted DNA from each sample was processed following the manufacturer's protocol and hybridized on Affymetrix GeneChip Genome-Wide Human SNP Array 5.0® (Affymetrix, Santa Clara, CA, USA). The QC call rates, which is an index measuring the quality of a SNP microarray experiment, obtained from the FFPE samples were from 70.75% to 91.93%, with a mean of 80.72% (Table 1), which was comparable to the results from former cytogenetic studies using DNA extracted from FFPE samples(9–11).

Copy number change and LOH analyses (called here SNPaCGH) were conducted using the Partek Genomics Suite (PGS) version 6.3 (Partek, St. Louis, MI, USA). To estimate the total rate of a copy number changed region, each segment amplified or lost was summed and divided by 2,829 Mb, which is the total Mb in the genome, excluding heterochromatic, centromeric, and telomeric regions not covered by probes. Similarly, to estimate the total rate of genomic alteration, the sum of segments with copy number change and copy number neutral loss of heterozygosity (CNLOH) was divided by 2,829 Mb. To validate the