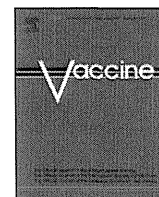Gur, R.E., Turetsky, B.I., Cowell, P.E., Finkelman, C., Maany, V., Grossman, R.I., Arnold, S. E., Bilker, W.B., Gur, R.C., 2000. Temporolimbic volume reductions in schizophrenia. Arch. Gen. Psychiatry 57 (8), 769–775.

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Contents lists available at SciVerse ScienceDirect

Vaccine

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The combined measles, mumps, and rubella vaccines and the total number of vaccines are not associated with development of autism spectrum disorder: The first case–control study in Asia

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ARTICLE INFO

ABSTRACT

Objective: The aim of this study was to investigate the relationship between autism spectrum disorder (ASD) and general vaccinations, including measles–mumps–rubella (MMR) vaccine, in Japanese subjects, a population with high genetic homogeneity.

Patients and methods: A case–control study was performed. Cases ($n = 189$) were diagnosed with ASD, while controls ($n = 224$) were volunteers from general schools, matched by sex and birth year to cases. Vaccination history and prenatal, perinatal, and neonatal factors from the Maternal and Child Health handbook, which was part of each subject's file, were examined. To determine the relationship between potential risk factors and ASD, crude odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated, and the differences in mean values of the quantitative variables between cases and controls were analyzed using an unpaired t-test. Moreover, MMR vaccination and the effect of the number of vaccine injections were investigated using a conditional multiple regression model.

Results: For MMR vaccination, the OR was 1.04 (95% CI, 0.65–1.68), and no significant differences were found for the other vaccines. For all of the prenatal, perinatal and neonatal factors, there were no significant differences between cases and controls. Furthermore, regarding the presence of ASD, MMR vaccination and the number of vaccine injections had ORs of 1.10 (95% CI, 0.64–1.90) and 1.10 (95% CI, 0.95–1.26), respectively, in the conditional multiple regression model; no significant differences were found.

Conclusions: In this study, there were not any convincing evidences that MMR vaccination and increasing the number of vaccine injections were associated with an increased risk of ASD in a genetically homogeneous population. Therefore, these findings indicate that there is no basis for avoiding vaccination out of concern for ASD.

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1. Introduction

Autism is a life-long neurodevelopmental disorder. Its prevalence was long considered to be approximately 4 in 10,000 [1]. Due to broadening of the nosological categorization and more widespread recognition, however, in recent years the prevalence of autism spectrum disorder (ASD) [2,3], which includes autism, Asperger syndrome, and Pervasive developmental disorder not

otherwise specified, has been reported at approximately 1% worldwide [4–6]. Although the pathogenesis of ASD has not yet been elucidated, genetic risk factors are strongly implicated, because the relative risk (λ_s) among siblings is greater than 20, and heritability is estimated to be as high as 38–90% [7–9]. In contrast, because the concordance rate of identical twins is not 100%, one can infer that environmental factors are also involved, and the recent increase in prevalence also indicates the involvement of various types of "novel environmental exposure". A debate has arisen over the contribution of vaccination as one environmental trigger of ASD.

The view that vaccination and ASD onset are related dates back to 1998 when the Lancet article by Wakefield et al. appeared [10] (the paper was retracted in 2010 because of ethical and methodological problems [11]). Thereafter, other published reports suggested a link between the measles–mumps–rubella vaccine

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(MMR) and ASD [12–14], and concerns emerged that thimerosal, which is included in other vaccines as a preservative, and vaccination with combined vaccines might be risks for ASD onset [15–17]. Other studies, however, that examined retrospective data and rejected any such link were published in rapid succession [18–26]. For example, some reported an increase in ASD prevalence despite a decline in the MMR vaccination rate [27,28]. In Japan, only two reports have been based on a time-series design, and the results suggested no relationship between MMR and ASD [29,30]. The most prominent articles in the past have focused mainly on the results of ecologic studies, and we will discuss the few existing case–control studies [31–33]. Each study demonstrated no differences between ASD cases and controls, failing to support a conclusion that immunization using MMR increases the risk of ASD onset.

Worldwide, reports on studies of immunization with vaccines other than MMR are rare. Moreover, parents or legal guardians remain apprehensive about the perceived risk of ASD posed by vaccination [34–37]. Therefore, the purpose of this study was to investigate Japanese subjects, a genetically homogeneous population, regarding links between ASD and immunization with various vaccines, including MMR, as well as the association between ASD and the number of vaccine injections [38]. This is the first case–control study in Asia investigating links between vaccination and ASD onset. These links were examined in ASD cases and controls matched for sex and year of birth based on data found in the Maternal and Child Health (MCH) handbook. This handbook, provided to all mothers by the relevant Japanese health system institution, is a highly reliable record of early development, health, and immunization, and health professionals (e.g. public health nurses, obstetricians, and pediatricians) keep record of most of the data listed in it [39,40]. In this study, therefore, data from the MCH handbook in terms of vaccination history, as well as potential prenatal, perinatal, and neonatal risk factors, were examined.

2. Patients and methods

2.1. Study population

2.1.1. Cases (Fig. 1)

The study analyzed case data from patients of the Yokohama Psycho-Developmental Clinic (YPDC), Kanto area, Japan, which accepts only patients with suspected developmental disorders. Of the patients who initially consulted the YPDC from April 1997 (opening of the clinic) until March 2011, the cases consisted of patients who: (1) were diagnosed with ASD, and (2) had been born between April 1, 1984 and April 30, 1992, the possible time period for MMR vaccination. Subjects whose records in the MCH handbook were missing or illegible and those with a history of vaccination in another country were excluded.

2.1.1.1. Diagnosis of ASD. Patients were diagnosed based on the classifications of pervasive developmental disorders in the Diagnostic and Statistical Manual 4th edition (DSM-IV) and standardized criteria using the Diagnostic Interview for Social and Communication Disorder (DISCO) [41,42]. The DISCO is recognized as one of the best ways to obtain a reliable and valid diagnosis of ASD [43].

One of several child psychiatrists on the team met the patient's parents and used the DISCO to take the patient's developmental history. Another child psychiatrist or clinical psychologist conducted intellectual or developmental tests, such as the Psycho-Educational Profile-Revised and Wechsler Intelligence Scale for Children-Third Edition. After the interview and testing, the diagnosis was made by the team according to the DSM-IV criteria.

2.1.1.2. Period of birth. MMR vaccination in Japan was conducted under specific circumstances. It was introduced in April 1989, and only one vaccination using MMR was included in the immunization schedule. The monovalent mumps and rubella vaccines remained the optimal choice of vaccine for those who did not participate in the MMR program. However, soon after the immunization program commenced, there were several cases of aseptic meningitis, which may have been caused by the mumps vaccine [44]. As a result, in April 1993, the Japanese government ceased extensive inoculation with MMR. Therefore, children born from April 1984 to April 1992 could receive the MMR vaccination, and those children were included in the present study.

2.1.2. Controls (Fig. 1)

One to two controls were selected for each case, matched by sex and year of birth and recruited as volunteers from general schools in the Kanto area, the same area where YPDC patients reside. Consent for participation in the present study was obtained from the parents (or legal guardians) of the students. Students who had previously been recognized as having developmental problems and were already receiving care were excluded, as were those whose records in the MCH handbook were missing or illegible and those with a history of vaccination in another country.
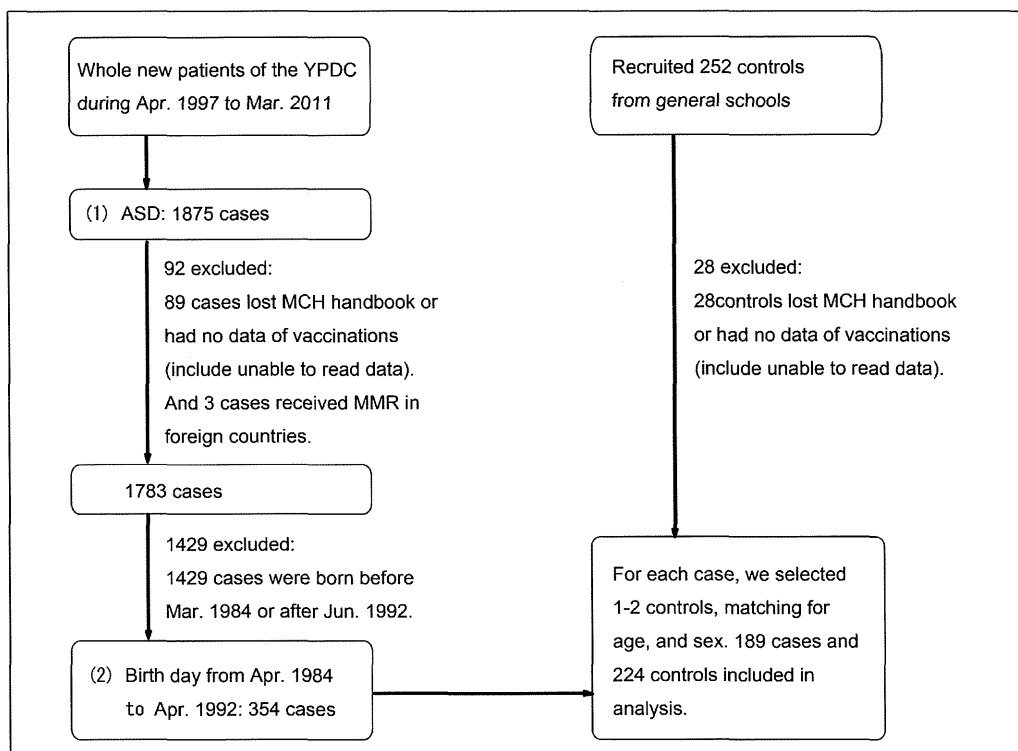
2.2. Source of data

The vaccination history and potential prenatal, perinatal, and neonatal risk factors collected based on the MCH handbook, which was routinely attached to each patient's file, were examined. The targeted vaccines were the MMR, generally used for infants, and the individual vaccines of the same type: the diphtheria–pertussis–tetanus vaccine (DPT); the polio vaccine; the B-encephalitis vaccine; and the Bacillus of Calmette and Guerin vaccine (BCG). For DPT, Polio, and B-encephalitis, there were many subjects who received these vaccines more than once. Therefore, the times of exposure to these vaccines were counted within the period of the first three years, when ASD features first appeared. Maternal hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg), albuminuria or edema, and anemia were examined as prenatal factors. The birth weight, head and chest circumference, duration of labor, delivery method (normal delivery, cesarean section, and obstetrical vacuum extraction or forceps delivery), and Apgar score were examined as perinatal and neonatal factors. Hypertension, albuminuria or edema, and anemia were recorded using a two-category scale (yes/no), and the Apgar score was recorded as an ordinal variable. Duration of labor, birth weight, and head and chest circumference were handled as continuous variables. The delivery method was recorded using a two-category scale (performed/not performed) for each delivery technique.

2.3. Selection of case children and matched control children

Among the patients who initially consulted the clinic between April 1997 and March 2011, 1875 cases of ASD were identified. Of these, 89 cases were excluded because the MCH handbook was missing or the vaccination record in the handbook could not be read, and 3 were excluded because they had received MMR vaccination overseas. Of the remaining 1783 cases, 1429 were born before March 1984 or after May 1992, leaving 354 cases (males: $n = 286$, 80.8%) born between April 1984 and April 1992, the possible time period for MMR vaccination. The ASD group consisted of 280 subjects with Autistic disorder (79.1%), 27 subjects with Asperger disorder (7.6%), and 47 subjects with Pervasive developmental disorder not otherwise specified (13.3%).

Numbers of potential cases and controls identified, excluded, and included in analysis.

Whole new patients of the YPDC during Apr. 1997 to Mar. 2011

Recruited 252 controls from general schools

(1) ASD: 1875 cases

92 excluded:
89 cases lost MCH handbook or had no data of vaccinations (include unable to read data). And 3 cases received MMR in foreign countries.

28 excluded:
28controls lost MCH handbook or had no data of vaccinations (include unable to read data).

1783 cases

1429 excluded:
1429 cases were born before Mar. 1984 or after Jun. 1992.

For each case, we selected 1-2 controls, matching for age, and sex. 189 cases and 224 controls included in analysis.

(2) Birth day from Apr. 1984 to Apr. 1992: 354 cases

YPDC= Yokohama Psycho-Developmental Clinic. ASD= Autism spectrum disorder.

MCH handbook= Maternal and Child Health handbook. MMR= measles-mumps-rubella vaccine.

Fig. 1. Numbers of potential cases and controls identified, excluded, and included in analysis. YPDC, Yokohama Psycho-Developmental Clinic; ASD, autism spectrum disorder; MCH handbook, maternal and child health handbook; MMR, measles–mumps–rubella vaccine.

As controls, 252 subjects from the general school population were recruited into the present study. Of these, 28 cases were subsequently excluded because the MCH handbook was missing or the vaccination record could not be read. The goal was to have a matched control for each case. However, since there were not enough controls to match to all cases, 189 subjects were chosen randomly from the ASD group as a case group. The controls were individually matched to cases by age and sex. There were 189 cases, mean age 22.6 years (SD 2.2), and 224 controls, mean age 22.6 years (SD 2.2), with case-to-control ratios ranging from 1:1 to 1:2 (Fig. 1).

2.4. Statistical analysis

2.4.1. Analysis 1

Duration of labor was divided into 2 categories of normal (≤ 20 h) versus prolonged labor (>20 h). Because an Apgar score of less than 7 has been associated with increased ASD risk [45–48], the Apgar score was divided into 2 categories of normal (≥ 7 points) and low (<7 points). In order to compare the backgrounds of the cases and controls, the crude odds ratios (ORs) and 95% confidence intervals (CIs) were determined for each outcome. The relationship between ASD onset and the total number of vaccine injections was also investigated. The crude ORs and 95% CI were determined for each. The differences in the mean values of the quantitative variables between cases and controls were examined by an unpaired t-test. When necessary, the t-test was modified for unequal variances.

2.4.2. Analysis 2

Because this study was only concerned with the theoretical increase in the risk of ASD onset due to the MMR vaccine injection, a conditional logistic model was applied to evaluate the ORs of MMR vaccination after adjusting for other risk factors.

2.4.3. Analysis 3

The OR of the total number of vaccine injections after adjusting for other risk factors was evaluated with the conditional logistic model.

2.4.4. Power analysis

Power analysis was performed in accordance to general power calculation model for chi squared statistics, t-test, and a conditional multiple regression model. In brief, power is determined with respect to degree of freedom and predefined alpha level of the study (0.05), number of predictors (in case of a conditional multiple regression model, 4) after assuming effect size (in accordance with Cohen's criteria).

Analysis 1 was performed using SPSS 17.0 for Japan, and Analyses 2 and 3 used the HALBAU 7. ORs were considered significant when the lower 95% CI exceeded 1.0. The t-tests were two-sided, and significance was defined as $p < 0.05$. For power calculation G*power v3.1 was used.

2.5. Ethical considerations

This study was approved by the ethics committee at Nagoya University. All data used in this study were clinical data obtained in the course of conventional diagnosis and therapy, and cooperation in the study placed no burden on individual patients. The parents or legal guardians of all of the children in the control group provided their written, informed consent to participate. Personal information regarding subjects in this study and the resulting data

Table 1

The proportions and crude odds ratios (ORs) and 95% confidence intervals (CI) for ASD according to vaccines, prenatal factors, perinatal factors and neonatal factors.

Variable category	n (%)		ORs	95% CI	p-Value
	Cases (n = 189)	Controls (n = 224)			
Vaccines					
MMR	47 (24.9)	54 (24.1)	1.04	0.65–1.68	.86
Measles	126 (66.7)	141 (62.9)	1.18	0.77–1.80	.43
Mumps	110 (58.2)	110 (49.1)	1.44	0.96–2.17	.06
Rubella	108 (57.1)	120 (53.6)	1.16	0.77–1.74	.47
DPT	185 (97.9)	219 (97.8)	1.06	0.24–4.75	.94
Polio	184 (97.4)	221 (98.7)	0.5	0.09–2.43	.73
B-encephalitis	167 (88.4)	206 (92.0)	0.66	0.33–1.34	.22
BCG	182 (96.3)	218 (97.3)	0.72	0.21–2.42	.55
Prenatal factors					
Maternal hypertension	6 (3.2)	3 (1.3)	2.42	0.53–12.36	.20
Albuminuria, edema	18 (9.5)	19 (8.5)	1.13	0.55–2.35	.71
Anemia	59 (31.2)	69 (30.8)	1.02	0.66–1.58	.93
Perinatal and neonatal factors					
Prolonged labor (>20 h)[a]	8/169 (4.7)	9/200 (4.5)	1.06	0.36–3.06	.92
Method of delivery					
Cesarean section	21 (11.1)	24 (10.7)	1.04	0.54–2.02	.90
Obstetrical vacuum extraction or forceps delivery[a]	23/168 (13.7)	22/200 (11.0)	1.28	0.66–2.51	.43
Low Apgar score (<7)	6 (3.2)	2 (0.9)	3.64	0.66–26.39	.09

ORs, odds ratios; CI, confidence intervals; ASD, autism spectrum disorder; MMR, measles–mumps–rubella vaccines; DPT, diphtheria–pertussis–tetanus vaccines; BCG, Bacillus of Calmette and Guerin vaccine.

[a] There were 20 cases and 24 controls who did a cesarean section, and 1 case who did a cesarean section because of prolonged labor. Thus, They were excluded from population of prolonged labor and obstetrical vacuum extraction or forceps delivery.

were rendered anonymous, and analyses were performed using only quantitative data that could not be linked to any particular subject.

3. Results

3.1. Vaccination rate and time of exposure to vaccines

The vaccination rates in cases and controls were as follows: MMR, 24.9% of cases and 24.1% of controls; Measles, 66.7% and 62.9%; Mumps, 58.2% and 49.1%; Rubella, 57.1% and 53.6%; DPT, 97.9% and 97.8%; Polio, 97.4% and 98.7%; B-encephalitis, 88.4% and 92.0%, and BCG 96.3% and 97.3% (Table 1). The mean times of each vaccine injection in cases and controls were as follows: DPT, 3.8 times of cases and 3.7 times of controls; Polio, 1.9 times and 2.0 times; B-encephalitis, 1.7 times and 1.8 times (Table 2).

3.2. Analysis

3.2.1. Analysis 1

For each vaccination, the ORs of cases versus controls were as follows (no significant differences were found): MMR, 1.04 (95% CI, 0.65–1.68); Measles, 1.18 (95% CI, 0.77–1.80); Mumps, 1.44 (95% CI, 0.96–2.17); Rubella, 1.16 (95% CI, 0.77–1.74); DPT, 1.06 (95% CI, 0.24–4.75); Polio, 0.50 (95% CI, 0.09–2.43); B-encephalitis, 0.66 (95% CI, 0.33–1.34); and BCG, 0.72 (95% CI, 0.21–2.42). Maternal hypertension as a prenatal factor had an OR of 2.42 (95% CI, 0.53–12.36), but no significant difference was found between cases and controls. For the other factors as well, cases did not have

significantly higher ORs than controls. As a perinatal and neonatal factor, low Apgar score and obstetrical vacuum extraction or forceps delivery had an OR of 3.64 (95% CI, 0.66–26.39) and 1.28 (95% CI, 0.66–2.51), respectively, but no significant difference was found between cases and controls. No other perinatal and neonatal factors showed significant differences between cases and controls (Table 1).

A t-test was performed on the mean values of the times of exposure to DPT, Polio, and B-encephalitis, birth weight and head and chest circumference between cases and controls, and no significant differences were found ($p > 0.05$ for all). The minimum number of vaccine injections was 3, and the maximum was 13. The mean (standard deviation) number of vaccine injections of cases and controls was 11.4 (1.7) and 11.4 (1.7), respectively, and there was no significant difference between cases and controls ($t = 0.07, p = 0.94$) (Tables 2 and 3).

3.2.2. Analysis 2

Maternal hypertension, low Apgar score, and obstetrical vacuum extraction or forceps delivery, which had higher ORs in the results of Analysis 1, were investigated as confounding factors using a conditional multiple regression model. With regard to the presence of ASD, MMR had an OR of 1.10 (95% CI, 0.64–1.90), and maternal hypertension, low Apgar score, and obstetrical vacuum extraction or forceps delivery had ORs of 4.19 (95% CI, 0.46–38.57), 2.06 (95% CI, 0.18–22.12) and 0.98 (95% CI, 0.50–1.92), respectively. There were no significant differences (Table 4).

Table 2

The comparison of the times of vaccine injection between cases and controls.

Vaccines	Cases Mean (±SD)	Controls Mean (±SD)	p-Value
DPT	3.8 (±0.8)	3.7 (±0.7)	.78[a]
Polio	1.9 (±0.3)	2.0 (±0.3)	.34[b]
B-encephalitis	1.7 (±0.7)	1.8 (±0.6)	.06[b]

DPT, diphtheria–pertussis–tetanus vaccines.

[a] Student's t-test.
[b] Welch's t-test.

Table 3

The comparison of quantitative variables between cases and controls.

Variables	Cases Mean (±SD)	Controls Mean (±SD)	p-Value
Birth weight (g)	3085.7 (±454.1)	3109.4 (±479.0)	.62[a]
Head circumference (cm)	33.5 (±2.3)	33.6 (±3.0)	.88[b]
Chest circumference (cm)	32.3 (±2.2)	32.3 (±2.7)	.90[a]
The number of vaccine injections (shots)	11.4 (±1.7)	11.4 (±1.7)	.94[a]

[a] Student's t-test.
[b] Welch's t-test.

Table 4

Odds ratios and 95% confidence intervals of MMR vaccination injection analyzed with a conditional logistic model.

Factor		ORs (95% CI)	p-Value
MMR vaccination injection	(−)	1	
	(+)	1.10 (0.64–1.90)	.72
Maternal hypertension	(−)	1	
	(+)	4.19 (0.46–38.57)	.21
Low Apgar score	(−)	1	
	(+)	2.06 (0.18–22.12)	.57
Obstetrical vacuum extraction or forceps delivery	(−)	1	
	(+)	0.98 (0.50–1.92)	.96

ASD, autism spectrum disorder; MMR, measles–mumps–rubella vaccines; ORs, odds ratios; 95% CI, 95% confidence intervals.

3.2.3. Analysis 3

The number of vaccine injections had an OR of 1.10 (95% CI, 0.95–1.26) in a conditional multiple regression model using the same confounding factors as for Analysis 2, maternal hypertension (OR = 3.63, 95% CI, 0.40–33.19), low Apgar score (OR = 2.14, 95% CI, 0.19–23.78), and obstetrical vacuum extraction or forceps delivery (OR = 1.02, 95% CI, 0.52–1.99), and there was no significant difference between cases and controls (Table 5).

3.2.4. Power analysis

Regarding power analysis for chi square statistics and t-test, when effect size is set to medium (in accordance to Cohen's criteria), both samples that are characterized in our research had more than 80% power for detecting association, respectively. However, in case, size effect is set to small, calculated power were 52% at chi square statistics and 53% at t-test. Similarly, regarding a conditional multiple regression model, our sample had more than 80% of power for detecting association in case of medium effect size. However in case, size effect is set to small, calculated power was 56%.

4. Discussion

The three previous case–control studies focused on the relationship between ASD and MMR. Specifically, the investigation of DeStefano et al. was based on the Metropolitan Atlanta Developmental Disabilities Surveillance Program [31]; Smeeth et al. used data from the UK General Practice Research Database [32]; and DeWilde et al. examined the association using the UK Doctors' Independent Network Database [33]. The aforementioned studies

Table 5

Odds ratios of one measure and 95% confidence intervals of the number of vaccine injections analyzed with a conditional logistic model.

Factor		ORs (95% CI)	p-Value
The number of vaccine injections	(−)	1	
	(+)	1.10[a] (0.95–1.26)	.19
Maternal hypertension	(−)	1	
	(+)	3.63 (0.40–33.19)	.25
Low Apgar score	(−)	1	
	(+)	2.14 (0.19–23.78)	.54
Obstetrical vacuum extraction or forceps delivery	(−)	1	
	(+)	1.02 (0.52–1.99)	.96

ASD, autism spectrum disorder; ORs, odds ratios; 95% CI, 95% confidence intervals.

[a] OR of the number of vaccine injections means OR of increasing one injection of vaccine.

provided no epidemiological evidence for a causal association. The present study is the first case–control study in Asia investigating the relationship between a variety of vaccines including MMR and the risk of ASD onset.

These previous studies were conducted using relatively heterogeneous samples in terms of genetic makeup. Conversely, the Japanese population is thought to be highly homogenous on the genetic level (which gives us the opportunity to minimize the effect of population-specific risk factors that might interact with environmental exposures (i.e. immunization)), and almost all Japanese parents have an MCH handbook. The fact that highly reliable information concerning the pregnancy, perinatal, and neonatal periods is collected in the handbook was advantageous for conducting this research.

In this study, we could not find the evidence that MMR vaccination increases the risk of ASD onset. The present results support the findings from the previous case–control studies conducted in Caucasian populations. Furthermore, we could not find any evidences that other types of vaccines or a combined effect of multiple vaccines was associated with ASD onset. Therefore, this study did not support the theory that vaccinations should be avoided to reduce the risk of ASD onset. We should be more concerned about acquiring infectious diseases by avoiding vaccinations.

In the results of this study, the 95% CIs of vaccinations, especially DPT, Polio, and BCG had a wide range because of small power. The sample size was not large enough to absolutely exclude the possibility that DPT, Polio, and BCG vaccinations increased the risk of ASD onset. Additionally, there were no theories about an increase in the risk of ASD onset concerns with any single types of vaccine injection that were included in this study, other than the MMR vaccine. Then a conditional logistic model was applied not to DPT, Polio, and BCG, but to MMR which was more concerned with the risk of ASD onset. This study was limited to data from the MCH handbook, which from the viewpoint of conducting an investigation is a highly reliable vaccination data source. On one hand we believe we have obtained very reliable results. However, on the other hand, the information in this handbook does not include several factors which were known to increase the risk of ASD onset, such as parental age at birth, bleeding, birth order, previous fetal loss, maternal prenatal medication use exclusively for hypertension and maternal toxaemia which were included in this study [49], and coexisting conditions that may influence vaccinations received, for example cardiovascular disease, other physical diseases or anomaly, epilepsy, or allergy. We were not able to investigate such conditions in controls because of the nature of the data collection procedure, which involved community-based sampling. Moreover, the relationship is unclear between the time periods when ASD was diagnosed and when the child was vaccinated. It has been hypothesized that early exposure to thimerosal and immune globulin preparations influence neuropsychological deficits in children include ASD [16,50]. Additionally, it is possible that parents lost motivation regarding vaccination before ASD was diagnosed because of problems such as the child's inability to sit still or frequent tantrums. Even so, both groups showed a high vaccination rate for each of the vaccines, and because the main topic of this study was to investigate whether vaccination increases the risk of ASD onset, we believe these effects can be ignored.

In future studies on vaccination and ASD, investigations with larger sample sizes are expected, and we anticipate examining factors which were known to increase the risk of ASD onset, coexisting conditions that may influence vaccination, such as cardiovascular disease, other physical diseases or anomaly, epilepsy, or allergies, the age at ASD diagnosis and vaccines injection, and reasons why vaccinations were not performed. We also look forward to

prospective studies that include pregnancy, delivery, or even pre-conception factors that may be associated with ASD.

In this study, we could not find any convincing evidence that MMR vaccination and increasing the number of vaccine injections were associated with an increased risk of ASD in a genetically homogeneous population. If such an association exists, it is so rare that it could not be identified in this large regional sample. Therefore, our findings indicate there is no basis for avoiding vaccination out of concern for ASD. This study investigated the link between vaccination and the risk of ASD, but it does not guarantee the safety or efficacy of the vaccines. Adverse reactions from vaccines other than a link with ASD exist. Such adverse reactions must be studied, and safer and more effective vaccines must be developed. At one time in Japan, mumps vaccine in the MMR vaccine caused several cases of aseptic meningitis. We should continue to investigate the safety and efficacy of vaccines carefully and the biological features of ASD in greater depth to improve outcomes related to long-term function and quality of life.

Acknowledgments

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Functional Genetic Variation at the *NRGN* Gene and Schizophrenia: Evidence From a Gene-Based Case—Control Study and Gene Expression Analysis

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Genome-wide association and follow-up studies have reported an association between schizophrenia and rs12807809 of the *NRGN* gene on chromosome 11q24.2. We investigated the association of five linkage disequilibrium-tagging SNPs and haplotypes that cover the *NRGN* gene with schizophrenia in a Japanese sample of 2,019 schizophrenia patients and 2,574 controls to determine whether rs12807809 is the most strongly associated variant for schizophrenia in the vicinity of the *NRGN* gene. We found that the rs12807809–rs12278912 haplotype of the *NRGN* gene was associated with schizophrenia (global $P = 0.0042$). The frequencies of the TG and TA haplotypes of rs12807809–rs12278912 in patients were higher (OR = 1.14, $P = 0.0019$) and lower (OR = 0.85, $P = 0.0053$), respectively, than in the controls. We did not detect any evidence of association of schizophrenia with any SNPs; however, two nominal associations of rs12278912 (OR = 1.10, $P = 0.057$) and rs2075713 (OR = 1.10, $P = 0.057$) were observed. Furthermore, we detected an association between the rs12807809–rs12278912 haplotype and *NRGN* expression in immortalized lymphoblasts derived from 45 HapMap JPT subjects ($z = 2.69$, $P = 0.007$) and confirmed

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the association in immortalized lymphoblasts derived from 42 patients with schizophrenia and 44 healthy controls ($z = 3.09$, $P = 0.002$). The expression of the high-risk TG haplotype was significantly lower than the protective TA haplotype. The expression was lower in patients with schizophrenia than in controls; however, this difference was not statistically significant. This study provides further evidence of the association of the NRGN gene with schizophrenia, and our results suggest that there is a link between the TG haplotype of rs12807809–rs12278912, decreased expression of NRGN and risk of developing schizophrenia. © 2012 Wiley Periodicals, Inc.

Key words: schizophrenia; neurogranin (NRGN); single nucleotide polymorphism (SNP); genome-wide association study (GWAS); gene expression

INTRODUCTION

Schizophrenia is a common and complex psychiatric disease with strong genetic components. Schizophrenia has an estimated heritability of approximately 80% [Cardno and Gottesman, 2000; Tsuang, 2000], and many genes have been implicated in the pathogenesis of schizophrenia [Sun et al., 2008].

Genome-wide association studies (GWAS) of single nucleotide polymorphisms (SNPs) investigate thousands of DNA samples from patients and controls, and these studies are a powerful tool for identifying common risk factors in complex diseases. Stefansson et al. [2009] combined the samples (from 12,945 patients with schizophrenia and 34,591 controls) from three large GWAS (the SGENE-plus, the International Schizophrenia Consortium and the Molecular Genetics of Schizophrenia GWAS) and conducted follow-up studies in 4,999 patients and 15,555 controls from four sets of samples from Europe, including from the Netherlands, Denmark, Germany, Hungary, Norway, Russia, Sweden, Finland, and Spain. The authors detected several significant association signals. Seven markers gave P values smaller than the genome-wide significance threshold of approximately 1.6×10^{-7} in the combined samples: five markers, rs6913660, rs13219354, rs6932590, rs13211507, and rs3131296, which spanned the major histocompatibility complex (MHC) region on chromosome 6p21.3-22.1; a marker, rs12807809, located 3,457 bases upstream of the neurogranin (NRGN) gene on chromosome 11q24.2; and a marker, rs9960767, in intron 4 of the transcription factor 4 (TCF4) gene on chromosome 18q21.2. Of the seven SNPs, four SNPs, rs6913660, rs13219354, rs13211507, and rs9960767, were not polymorphic in the HapMap Japanese in Tokyo (JPT) samples. The minor allele frequencies (MAFs) for two SNPs, rs6932590 and rs3131296, were less than 5%. Because only one marker, rs12807809, in the NRGN gene was a common SNP in the HapMap JPT samples (MAF greater than 5%), we focused on this SNP and the NRGN gene in the present study.

The NRGN gene is the human homolog of the neuron-specific rat RC3/neurogranin gene. NRGN encodes a postsynaptic protein kinase substrate that binds calmodulin (CaM) in the absence of calcium and has been implicated in dendritic spine formation and synaptic plasticity [Baudier et al., 1991]. NRGN plays an important role in the Ca^{2+}–CaM signaling pathway [Hayashi, 2009]. Ca^{2+} influx-induced oxidation of NRGN leads to the postsynaptic activation of CaM-dependent protein kinase II (CaMKII) by CaM, which is associated with strengthened N-methyl-D-aspartate (NMDA) receptor signaling [Li et al., 1999]. Reduced NRGN activity may mediate the effects of NMDA hypofunction implicated in the pathophysiology of schizophrenia.

The NRGN gene spans 7.3 kb of genomic DNA and contains four exons [Martinez de Arrieta et al., 1997]. Part of exon 1 and exon 2 encode a 78-amino-acid protein, and exons 3 and 4 contain untranslated sequences. A thyroid hormone response element (TRE) has been identified in intron 1 [Martinez de Arrieta et al., 1999]. An association between the NRGN gene and schizophrenia has previously been reported in a small population of male Portuguese and Brazilians [Ruano et al., 2008], although the associated SNP in the study, rs7113041, was not tightly correlated with the genome-wide supported SNP, rs12807809 (HapMap CEU $r^2 = 0.07$, JPT $r^2 = 0.01$). In addition, two separate studies reported no association between the genetic variants of NRGN and schizophrenia in Bulgarian [Betcheva et al., 2009] and Chinese populations [Li et al., 2010]. The genome-wide supported SNP and other SNPs in the NRGN gene were not genotyped in the GWAS of schizophrenia in Japanese populations because of a difference in the genotyping chips used among the separate GWAS, which the Illumina HumanHap 300 or 550 BeadChips, Affymetrix Genome-Wide Human SNP Array 5.0 and Affymetrix GeneChip Mapping 100 K microarrays [Stefansson et al., 2009; Ikeda et al., 2011; Yamada et al., 2011] were used. Here, we first investigated the association between the NRGN gene and schizophrenia in a Japanese population using a gene-based approach to determine whether rs12807809 is the most strongly associated variant for schizophrenia near the NRGN gene. Second, we examined whether the associated haplotype of NRGN influenced NRGN expression in immortalized lymphoblasts derived from the HapMap JPT samples and our Japanese case–control samples.

MATERIALS AND METHODS

Subjects

Subjects for the genetic association analysis included 2,019 unrelated patients with schizophrenia (54.5% males, with a mean age ± SD of 44.7 ± 15.1 years) and 2,579 unrelated healthy controls (49.4% males, 45.4 ± 19.4 years). The mean age did not differ significantly between cases and controls ($P = 0.24$); however, the male to female ratio of the patients was significantly higher than in the controls ($P < 0.05$). Age and sex-matched subjects for NRGN expression analysis consisted of 42 patients with schizophrenia (58.1%, 38.4 ± 11.2 years) and 44 healthy subjects (56.8% males, 38.0 ± 11.4 years). These subjects were included in the genetic association analysis. All subjects used in both analyses were biologically unrelated, of Japanese ethnicity and were recruited from four geographical regions in Japan: Osaka, Aichi, Tokushima, and Tokyo [Yamaguchi-Kabata et al., 2008; Ohi et al., 2009]. Cases were recruited from outpatient and inpatient facilities at university hospitals and psychiatric hospitals. Each subject with schizophrenia had been diagnosed by at least two trained psychiatrists based on an unstructured clinical interview; diagnoses were made based on the

criteria of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Controls were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had current or past contact with psychiatric services. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Fujita Health University, Nagoya University, Tokushima University and Juntendo University.

SNP Selection and SNP Genotyping

This study was designed to examine the association between the NRGN gene and schizophrenia by selectively tagging SNPs in the NRGN gene and flanking regions (±5 kb). We selected five tagging SNPs using the TAGGER algorithm (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger) with the criteria of r^2 greater than 0.80 in "pair-wise tagging only" mode and an MAF greater than 5%, which was implemented in Haploview 4.2 using HapMap data release 27 Phase II + III, Feb 2009, on NCBI B36 assembly, dbSNP b126 [Japanese in Tokyo (JPT), Chr 11: 124,109,952.124,127,307]. The five tagging SNPs were rs1939214, rs12807809, rs12278912, rs2075713, and rs11219769. Markers are shown in Table I; orientation and alleles are reported on the genomic plus strand (rs12807809 is reported as T/C, as has been reported in previous GWAS [Stefansson et al., 2009]). Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, CA) as previously described [Hashimoto et al., 2006, 2007]. Detailed information on the PCR conditions is available upon request. Genotyping call rates were 98.9% (rs1939214), 98.5% (rs12807809), 99.3% (rs12278912), 99.3% (rs2075713), and 99.5% (rs11219769). No deviation from Hardy–Weinberg equilibrium (HWE) in the examined SNPs was detected in the patients with schizophrenia or healthy controls ($P > 0.05$). The positions of the five SNPs analyzed in the present study are shown in Figure 1.

Quantitative Measurement of NRGN Gene Expression

Isolation and immortalization procedures of lymphocytes from blood using the Epstein–Barr virus (EBV) were performed by SRL of Tokyo, Japan. Immortalized, patient-derived lymphocytes were grown in culture media supplemented with 20% fetal bovine serum. Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). The total yield of RNA was determined by absorbance at 260 nm, and the quality of the RNA was determined using agarose gel electrophoresis.

According to the manufacturer's protocol, total RNA was treated with DNase to remove contaminating genomic DNA using DNase Treatment and Removal Reagents (Ambion, Austin, TX). Total RNA (10 μg) treated with DNase was used in a 50-μl reverse transcriptase reaction to synthesize cDNA with the SuperScript

TABLE I. Genotype and Allele Distributions for SNPs in the NRGN Gene Between Patients With Schizophrenia and Controls in a Japanese Population

Marker				SCZ [n = 2019]			CON [n = 2579]			Genotypic P-value [χ²]	MAF		Allelic P-value [χ²]	OR [95% CI]
SNP IDs [M]	Position[a]	M/m[b]	Gene	M/M	M/m	m/m	M/M	M/m	m/m		SCZ	CON		
rs1939214 [A]	124110500	A/G	5′	0.67	0.30	0.04	0.66	0.30	0.04	0.51 [1.3]	0.19	0.19	0.29 [1.1]	1.06 [0.95–1.18]
rs12807809 [T][c]	124111495	T/C	5′	0.58	0.35	0.07	0.56	0.37	0.07	0.44 [1.6]	0.25	0.26	0.25 [1.3]	1.06 [0.96–1.16]
rs12278912 [G][d]	124117369	G/A	Intron1	0.61	0.34	0.05	0.59	0.35	0.06	0.13 [4.1]	0.22	0.23	0.057 [3.6]	1.10 [1.00–1.22]
rs2075713 [A]	124123149	A/C	3′	0.65	0.31	0.04	0.62	0.33	0.05	0.17 [3.5]	0.20	0.21	0.057 [3.6]	1.10 [1.00–1.22]
rs11219769 [G]	124125357	G/T	3′	0.57	0.37	0.06	0.55	0.38	0.07	0.24 [2.8]	0.25	0.26	0.09 [2.8]	1.09 [0.99–1.19]

SCZ, patients with schizophrenia; CON, healthy controls; M, major allele; m, minor allele; MAF, minor allele frequency; OR, odds ratio.
[a] db SNP build 129.
[b] The first alleles shown are major alleles. All the alleles are represented according to the plus strand DNA sequence.
[c] The genome-wide supported SNP for schizophrenia [Stefansson et al., 2009].
[d] Because a high linkage disequilibrium between rs12278912 and rs7113041 [Ruano et al., 2008] was found in the HapMap JPT samples ($r^2 = 0.93$), rs12278912 was selected as the tagging SNP by the TAGGER program.
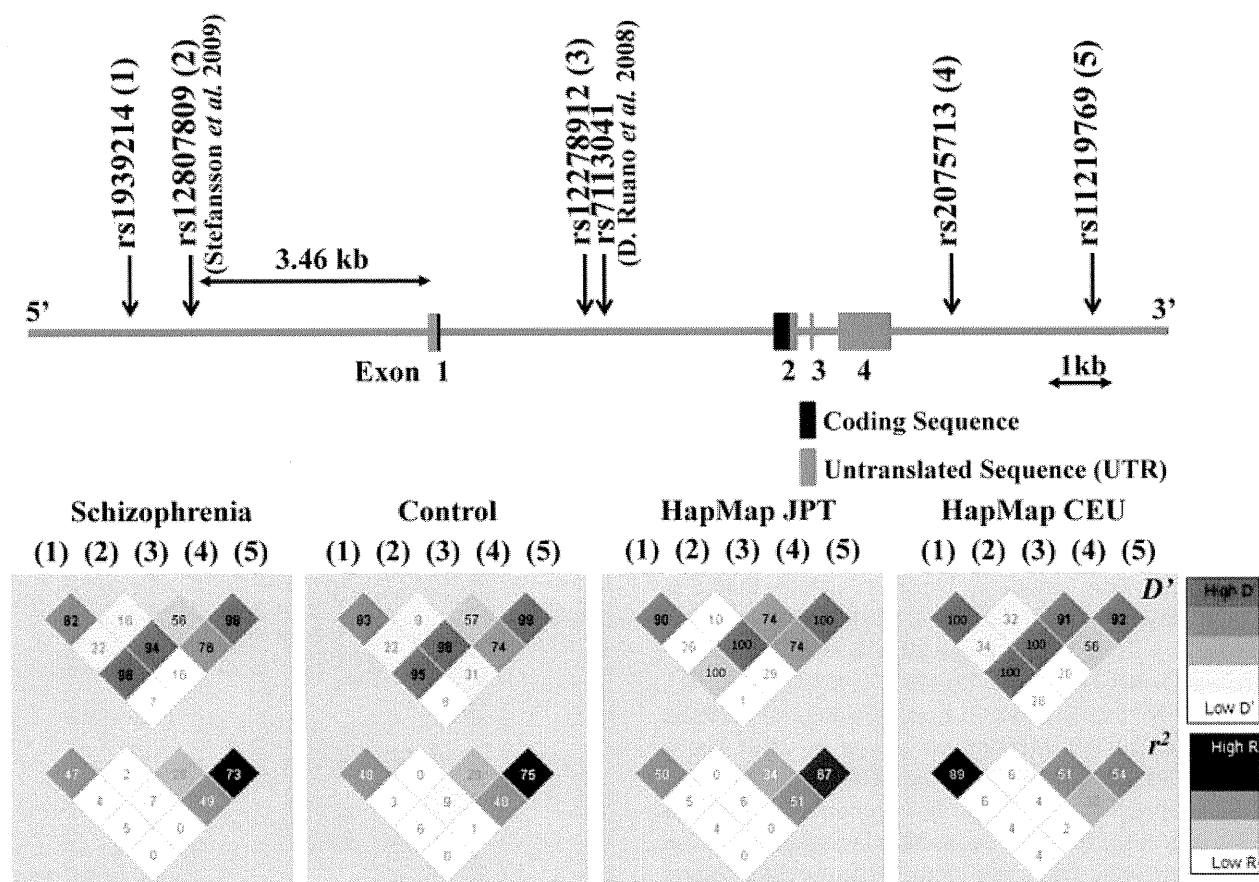
FIG. 1. The genomic structure of *NRGN*, including the locations of the five tagging SNPs studied and linkage disequilibrium of these SNPs in the patient, control, HapMap JPT, and CEU groups. Based on an entry in the Entrez Gene database (National Center for Biotechnology Information), the genomic structure of *NRGN* is shown above. The locations of the SNPs analyzed in this study are indicated by arrows, with numbers indicated in parentheses. The numbers indicated in parentheses refer to the numbering of the SNPs in the linkage disequilibrium (LD) diagram. The distances of exons—introns and intermarkers are drawn to scale. The LDs between pairwise SNPs are shown using the D′ (upper) and r^2 (lower) values at the bottom of the map of the gene structure separately for cases, controls, the HapMap JPT samples and the HapMap CEU samples. High levels of LD are represented by black (D′ and r^2) coloring, with increasing color intensity from 0 to 100, as shown by color bars.

First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Detailed information on the PCR conditions is available upon request.

To measure mRNA expression levels of housekeeping (β-*actin*) and *NRGN* genes, we used the Pre-Developed TaqMan Assay Reagent kit (Applied Biosystems). Primer information (gene name: assay ID, transcript ID, target region) is as follows; *NRGN*: Hs00382922_m1, NM_001126181.1 and NM_006176.2, Exon1-2; β-actin: 4326315E, NM_001101, no region indicated (Applied Biosystems). Expression levels of these genes were measured by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with a 384-well format as previously described [Yamamori et al., 2011; Yasuda et al., 2011]. PCR data were obtained using Sequence Detector software (SDS version 2.1; Applied Biosystems) and quantified using a standard curve. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon

profile. The software plotted a standard curve of the cycle at threshold C_t, which is where the fluorescence generated within a reaction crossed the threshold, versus the quantity of RNA. All samples were measured using a single plate per target gene, and their C_t values were in the linear range of the standard curve. The quantity of each sample was predicted by C_t values. The qRT-PCR reaction was performed in triplicate, and the expression level of the gene was taken as the average of three independent measurements. Standard curves were obtained using serial dilutions (1:4) of pooled complementary DNA prepared from 300 ng total RNA derived from immortalized lymphocytes. The standard curves of β-*actin* and *NRGN* showed that these genes were expressed in immortalized lymphocytes. In each experiment for β-*actin* and *NRGN*, the R^2 value of the standard curve was >0.99, and no-template control assays resulted in no detectable signal. The individual expression levels of the *NRGN* gene were normalized to the housekeeping gene (raw target gene expression level divided by raw housekeeping gene expression level) and were used for statistical analysis.

Haplotype Associated With *NRGN* Expression (eQTL)

To identify whether the haplotypes in *NRGN* associated with schizophrenia may be expression quantitative trait loci (eQTL), we analyzed *NRGN* expression in two datasets of lymphoblast-derived HapMap JPT samples and in the Japanese case–control samples. For the HapMap JPT samples, we extracted genotypes and *NRGN* lymphoblastoid expression data from the HapMap JPT samples (n = 45) deposited in GeneVar (http://www.sanger.ac.uk/humgen/genevar/ [Stranger et al., 2007]). For the Japanese case–control samples, we used our genotypes and *NRGN* lymphoblastoid expression data obtained using the method described above.

Statistical Analyses

We performed power calculations using the Power Calculator for Two-Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/ [Skol et al., 2006]). The power estimate was based on an allele frequency of 0.83 at rs12807809, an odds ratio of 1.19, which was indicated by Stefansson et al. [2009], a prevalence of 0.01, and an alpha level of 0.05 using a multiplicative model.

Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2 tests for sex and the Mann–Whitney U-test for age using PASW Statistics 18.0 software (SPSS Japan, Inc., Tokyo, Japan). Deviation from HWE was tested separately in test cases and controls using χ^2 tests for goodness of fit using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The allelic and genotypic distributions of *NRGN* polymorphisms between patients and controls were analyzed using χ^2 tests with SNPAlyze V5.1.1 Pro software. The number of effective independent SNPs assayed was estimated to correct for multiple testing by the spectral decomposition method of Nyholt using the SNPSpD software [Nyholt, 2004]. The effective number of independent marker loci was 4.13 and corrected P-value for allelic and genotypic associations was set at $P < 0.012$. Pairwise linkage disequilibrium (LD) analyses expressed by D′ and r^2 were applied to detect the intermarker relationships in each group using Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated using the method of maximum likelihood with genotyping data using the expectation–maximization (EM) algorithm from SNPAlyze V5.1.1 Pro software. Rare haplotypes detected in less than 3% of patients and controls were excluded from the haplotypic association analysis, as previously described [Ohi et al., 2009, 2010]. We performed 10,000 permutations for significance tests to determine empirical significance using a 2 × 2 contingency table approach. We used a 2- to 5-window fashion analysis. Since Bonferroni correction for multiple testing is considered to be too conservative to apply to genetic association analyses [Nyholt, 2001], method of Nyholt [Nyholt, 2004] for allelic and genotypic associations and permutation tests [Dudbridge, 2003] for haplotypic associations are considered to be appropriate for these analyses.

The difference in expression levels between Japanese patients with schizophrenia and controls was analyzed using linear regression in PASW Statistics 18.0 software. Age and sex, which may influence gene expression, were corrected for in the expression analysis. HPlus (http://qge.fhcrc.org/hplus) is a software applica-tion for estimating haplotype frequencies and inferring individual haplotypes based on EM and progressive ligation (PL) algorithms [Li et al., 2003], and most significantly assessing haplotypic associations with various types of phenotypes using linear regression. Differences of expression levels among haplotypes were analyzed using linear regression in HPlus software. Each genotype was treated as the number of major alleles (0, 1, and 2) in the expression analysis. For the joint haplotype analysis in HPlus software, each haplotype was tested against the reference haplotype (equal to most frequent haplotype) using linear regression. As age and sex were not available for the HapMap samples, these confounding factors were not corrected for in the expression analysis. Expression levels in Japanese cases, control samples and in the combined samples were corrected for age and sex in the analyses. We applied a Bonferroni correction in expression analysis (three tests). The significance level for statistical tests was set at two-tailed $P < 0.05$.

RESULTS
Genetic Association Analysis

Our study size of 2,019 cases and 2,579 controls had sufficient power (>80%) to detect a genetic effect at ORs of 1.19 or greater for rs12807809 when the allele frequency was 0.83, as described in previous GWAS (SGENE-plus) [Stefansson et al., 2009].

The genotype and allele frequencies of five tagging SNPs located in the *NRGN* gene and flanking regions are summarized in Table I. There was no allelic or genotypic association with schizophrenia for any of the five SNPs (uncorrected $P > 0.05$). However, nominal differences in allele frequencies between patients and controls were observed in rs12278912 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$) and rs2075713 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$). The major allele frequencies of both SNPs were higher in patients than in controls. Consistent with previous GWAS [Stefansson et al., 2009], the frequency of the major T allele of rs12807809 was higher in patients (75.4%) than in controls (74.4%) in our Japanese population, although the results did not reach statistical significance [$\chi^2 = 1.3$, $P = 0.25$, OR (95% confidence interval (CI)) = 1.06 (0.96–1.16)].

We focused on haplotypic association between patients with schizophrenia and healthy subjects using a 2- to 5-window fashion analysis. Haplotype analysis showed a significant association with schizophrenia (rs12807809–rs12278912, $\chi^2 = 13.1$, global $P = 0.0042$) (Supplementary Table I). The frequency of the major TG haplotype of rs12807809–rs12278912 was higher in patients (62%) than in controls (58%) [$\chi^2 = 9.4$, $P = 0.0019$, OR (95% CI) = 1.14 (1.05–1.24)] (Table II). On the other hand, the frequency of the TA haplotype of rs12807809–rs12278912 was lower in patients (14%) than in controls (16%) [$\chi^2 = 7.3$, $P = 0.0053$, OR (95% CI) = 0.85 (0.76–0.96)] (Table II). There was no haplotypic association with schizophrenia for any other haplotypes. These findings suggest that the major TG haplotype of rs12807809–rs12278912 may be related to an increased risk of schizophrenia, and the TA haplotype may have a protective role against the susceptibility to schizophrenia. These results of allelic, genotypic, or haplotypic associations were not affected by excluding 86 samples used for expression analyses (data not shown).

TABLE II. Differences in the rs12807809–rs12278912 Haplotype Between Patients With Schizophrenia and Healthy Subjects

Haplotype rs12807809[a]–rs12278912[b]	Frequency		Individual P (χ^2)	OR (95%CI)	Global P (χ^2) 0.0042 (13.1)
	Patients	Controls			
TG	0.62	0.58	**0.0019 (9.4)**	1.14 (1.05–1.24)	
CG	0.17	0.18	0.07 (3.4)	0.90 (0.81–1.01)	
TA	0.14	0.16	**0.0053 (7.3)**	0.85 (0.76–0.96)	
CA	0.08	0.08	0.57 (0.3)	1.05 (0.90–1.22)	

Significant P values are shown as bold-faced and underlined type.
[a]The genome-wide supported SNP for schizophrenia [Stefansson et al., 2009].
[b]Because a high linkage disequilibrium between rs12278912 and rs7113041 [Ruano et al., 2008] was found in the HapMap JPT samples ($r^2 = 0.93$), rs12278912 was selected as the tagging SNP by the TAGGER program.

The LD relationships between the markers are provided in Figure 1. The LD pattern observed in our controls was similar to our patients and the JPT HapMap samples; however, it was different from that of the CEU HapMap samples. The strengths of the LD patterns of rs1939214–rs12807809 and rs12278912–rs2075713–rs11219769 were different between Japanese populations and the CEU HapMap samples. The low LD pattern of rs12807809–rs12278912 was similar among the groups ($D' < 0.50$, $r^2 < 0.10$).

NRGN Gene Expression Analysis

The NRGN expression level was lower in patients with schizophrenia (n = 42, mean ± SD, 0.86 ± 0.58) than in controls (n = 44, 1.00 ± 0.75). However, the results did not reach statistical significance (r = −0.14, beta = −0.11, SE = 0.14, t = −0.97, P = 0.34).

Based on the results from the genetic association analysis, we investigated whether the rs12807809–rs12278912 haplotype of the NRGN gene was an eQTL in two datasets. The rs12807809–rs12278912 haplotype related to schizophrenia was significantly associated with NRGN expression in healthy HapMap JPT samples. The NRGN gene expression of the high-risk TG haplotype of rs12807809–rs12278912 was significantly lower than that of the protective TA haplotype (z = 2.69, P = 0.007). We confirmed that the rs12807809–rs12278912 haplotype was significantly associated with NRGN expression normalized to the β-actin expression in the controls and combined samples (Fig. 2 and Table III, control samples: z = 2.30, P = 0.021, combined samples: z = 3.09, P = 0.002). The association occurred in the same direction among the HapMap JPT, control, and combined samples. In case samples, the expression level of rs12807809–rs12278912 was lower in samples with the high-risk TG haplotype than in those with the protective TA haplotype, although the result did not reach statistical significance (z = 1.49, P = 0.14). The association in the HapMap JPT and combined samples remained significant after correction for multiple tests (HapMap JPT samples: corrected P = 0.021, combined samples: corrected P = 0.006). However, there was no significant association after applying the correction in control samples (corrected P = 0.063).

DISCUSSION

In this study, we provided evidence that haplotypes, including the genome-wide-screen-supported SNP of the NRGN gene, were associated with an increased risk of schizophrenia. Our in silico analysis showed that the high-risk rs12807809–rs12278912 haplotype of the NRGN gene may be associated with a low expression level of the NRGN gene in lymphoblasts derived from the HapMap JPT samples. We confirmed the association between the haplotype and NRGN expression in the combined case–control samples. Our results suggest that the schizophrenia-associated haplotype at the
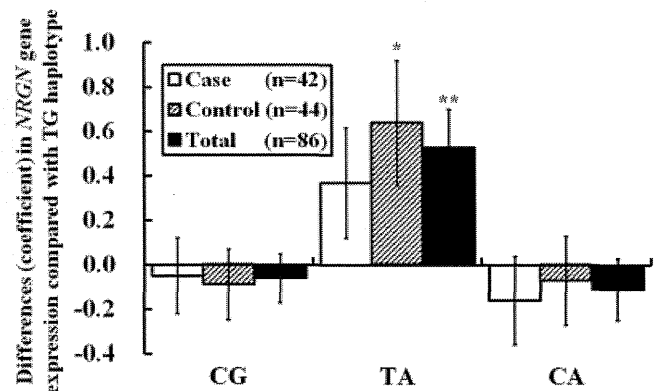

FIG. 2. The association between the rs12807809–rs12278912 haplotype of the NRGN gene and NRGN expression in lymphoblasts. Expression of the protective TA haplotype of rs12807809–rs12278912 was significantly higher than that of the high-risk TG haplotype in controls and combined case–control samples. The error bars represent standard errors of the coefficient. Estimated frequencies of each haplotype were as follows—TG haplotype: Case, 69%; Control, 61%; Total, 65%; CG haplotype: Case, 16%; Control, 20%; Total, 18%; TA haplotype: Case, 7%; Control, 11%; Total, 9%; CA haplotype: Case, 8%; Control, 9%; Total, 8%. *$P < 0.05$, **$P < 0.01$.

NRGN gene may be a functional variant, and the results support an association between the *NRGN* gene and schizophrenia.

This report is the first investigation of the association of tagging SNPs and haplotypes covering the *NRGN* gene with schizophrenia. To our knowledge, five genetic studies have investigated whether the *NRGN* gene is implicated in schizophrenia. A genome-wide linkage study has shown that the chromosomal region 11q23.3-24 including the *NRGN* gene is linked to schizophrenia in British and Icelandic populations [Gurling et al., 2001]. Subsequently, an association study determined that rs7113041, which displays high LD with rs12278912 and is located on intron 1 in the *NRGN* gene, is related to the risk of developing schizophrenia in male subjects of Portuguese origin [Ruano et al., 2008]. In addition, three GWAS and follow-up studies have shown that rs12807809 is associated with schizophrenia in large European samples [Stefansson et al., 2009]. However, two studies reported no association between *NRGN* and schizophrenia in Bulgarian or Chinese populations [Betcheva et al., 2009; Li et al., 2010]. In the present study, we determined that the rs12807809–rs12278912 haplotype is associated with an increased risk of schizophrenia in a Japanese population. However, there were no significant associations between any SNP, including rs12807809 and rs12278912, and schizophrenia in the population. The inconsistency of association among the previous studies and the present study might result from ethnic differences or type I or II errors for the different sample sizes: Portuguese, 315 cases, 295 controls and 73 trios [Ruano et al., 2008]; European Caucasian, 12,945 cases and 34,591 controls [Stefansson et al., 2009]; Japanese, 2,019 cases and 2,579 controls (present study); Bulgarian, 185 cases and 184 controls [Betcheva et al., 2009]; and Chinese, 2,496 cases and 5,184 controls [Li et al., 2010]. In addition, the SNPs investigated in each study were different. Ruano et al. [2008] and Betcheva et al. [2009] examined rs7113041, which has high LD with rs12278912 but not with rs12807809, whereas Stefansson et al. [2009] and Li et al. [2010] examined rs12807809

but not rs12278912. However, none of these studies examined haplotypes for the *NRGN* gene. Because the rs12807809–rs12278912 haplotype may be the most significant genetic variant in this region, further study is required to confirm the association between the rs12807809–rs12278912 haplotype and schizophrenia in other populations.

Differences in the relative *NRGN* expression levels between patients with schizophrenia and healthy subjects were not demonstrated. This result may be due to the small sample sizes in this study, which may have resulted in the failure to identify a modest difference in *NRGN* expression in this complex disease. We determined that the major TG haplotypic and the TA haplotypic frequencies of rs12807809–rs12278912 were higher and lower, respectively, in patients with schizophrenia than in healthy controls. In addition to these findings, we found that *NRGN* gene expression of the high-risk TG haplotype was significantly lower than that of the protective TA haplotype in lymphoblasts derived from our Japanese case–control subjects as well as the JPT HapMap sample. The low LD patterns of rs12807809–rs12278912 were similar across populations. This region may be vulnerable to recombination. Combinations of the TG and TA of rs12807809–rs12278912 could play an important role in the pathogenesis of schizophrenia. In this study, gene expression data derived from lymphoblasts raised the possibility that the rs12807809–rs12278912 haplotype may be a functional variant of *NRGN*. Further biological studies of the function of rs12807809–rs12278912 are required to verify the expression results.

Smith et al. [2011] analyzed *NRGN* expression in several brain tissues derived from a dataset of at least 130 individuals of European ancestry. However, they showed that neither the genome-wide supported SNP nor any individually correlated SNPs were associated with *NRGN* expression. They did not examine any association between haplotype and *NRGN* expression. There are several challenges in investigating expression findings in the postmortem

TABLE III. The Association Between the rs12807809–rs12278912 Haplotype and mRNA Expression

Haplotypes	Frequency	Coefficient	SE	CI	*P*-value (*Z*-score)
Schizophrenia (*n* = 42)					
TG	0.69	0 (ref)	—	—	—
CG	0.16	−0.05	0.17	(−0.39–0.29)	0.76 (−0.30)
TA	0.07	0.37	0.25	(−0.12–0.86)	0.14 (1.49)
CA	0.08	−0.16	0.20	(−0.55–0.24)	0.43 (−0.78)
Healthy control (n = 44)					
TG	0.61	0 (ref)	—	—	—
CG	0.20	−0.09	0.16	(−0.39–0.22)	0.58 (−0.55)
TA	0.11	0.64	0.28	(0.09–1.18)	**0.021 (2.30)**
CA	0.09	−0.07	0.20	(−0.46–0.32)	0.73 (−0.34)
Total subjects (n = 86)					
TG	0.65	0 (ref)	—	—	—
CG	0.18	−0.06	0.11	(−0.28–0.15)	0.57 (−0.57)
TA	0.09	0.53	0.17	(0.19–0.87)	**0.002 (3.09)**
CA	0.08	−0.11	0.14	(−0.39–0.17)	0.45 (−0.75)

Joint Association Analysis (the reference haplotype is the most frequent haplotype).
For the joint haplotype test, several haplotypes were tested against the reference haplotype.
Significant *P* values are shown as bold-faced and underlined type.

brain: (1) the choice of an appropriate brain region for investigation; (2) the heterogeneity of cell types within brain tissue; (3) the reliance on relatively small samples; and (4) the impact of cause of death and/or postdeath handling of the tissues on gene expression [Marcotte et al., 2003]. Thus, the use of postmortem brain tissue is compounded by a range of confounding factors (age, race, gender, different microarray platforms, and analysis methods) and may be the cause of the relative lack of gene/transcript-level consistency among expression studies. To overcome some of these problems, several groups have considered the use of lymphoblasts rather than the postmortem brain [Matigian et al., 2008; Slonimsky et al., 2010; Yamamori et al., 2011; Yasuda et al., 2011]. Lymphoblasts are useful for schizophrenia researchers because blood-based tissue (lymphoblasts) can be obtained with ease from living subjects, which allows larger case–control studies with optimal matching of key variables (age, sex, and race). In addition, immortalized lymphoblasts in culture are considered an effective tool for studying cells in the absence of the effect of antipsychotic treatments and duration of illness, both of which could mask the genetic differences in RNA expression. Thus, lymphoblasts could be good tool to investigate the impact of a gene in the absence of the impact of any confounding factors. On the other hand, there were some demerits of using lymphoblasts. In immortalized lymphocytes, it might be difficult to observe the effects of genes on their neuron-specific functions, for example, the effects of genes on glutamate and dopamine release and on the formation of synaptic vesicles. When isolation and immortalization procedures of lymphocytes from blood were performed or immortalized lymphocytes were grown in culture media, a genetic mutation might be inserted into genomic DNA in the cultured lymphoblasts and alter DNA sequences. It remains still controversial whether immortalized lymphocytes are an appropriate alternative to neuronal tissue, because there was a little evidence of analysis using immortalized lymphocytes from patients with schizophrenia. In this study, the difference in the association of gene expression with genetic variants between previous study and present study could be explained by the difference in the gene expression profile between immortalized lymphoblast and postmortem brain tissue. Other possible factors contributing to differences in association between studies could be a difference in the SNPs and haplotypes investigated or ethnic differences between Japanese and Caucasian populations.

Smith et al. [2011] performed mutation searches of all four exons of NRGN gene in 14 Caucasian subjects with schizophrenia and of the cording exons of NRGN gene in 1,113 Bulgarians individuals, 699 of whom had schizophrenia. However, they did not find any novel common polymorphism in the region. Thus, we did not perform a systematic mutation search in this study because there has been no novel common genetic variant in the region. If we perform sequencing and find a novel rare polymorphism, we cannot analyze association between the rare polymorphism and gene expression for only a small number of individuals with rare variant. A genetic variant, particularly a SNP not listed in the HapMap database, that is likely to be more strongly associated with schizophrenia may exist in the rs12807809–rs12278912 haplotype region. Sequencing the entire gene in individuals with risk haplotype in comparison with the protective haplotype carriers with larger sample sizes could provide further information underlying the genomic mechanism for this risk haplotype.

There are several limitations to interpreting our results. Because a number of statistical analyses supported the association of the NRGN gene and schizophrenia, such as genotypic and allelic associations for five SNPs (total 5×2), haplotype analysis using a window fashion analysis (total 10) and expression analysis for three individual haplotypes (total 3×4), a correction for multiple testing should be considered. In this study, the overall number of genetic association tests was 32; however, not all tests were independent, and several hypotheses were included. Thus, Bonferroni correction, a method to correct for multiple independent tests for one hypothesis, might not be appropriate. The consensus how to correct such multiple testing has not been reached in this research field. Thus, we applied SNPSpD correction for genotypic and allelic association analysis, permutation method for haplotype analysis and Bonferroni correction for expression analysis (three tests). However, even though we applied these methods of correcting such multiple testing, they might cause false positive results. We did not control for geographical variation of control origin because there is little possibility for ethnic/genetic difference among four geographical regions for feature of homogeneous race in Japan [Yamaguchi-Kabata et al., 2008]. Our significant results may be derived from sample bias owing to population stratification and non-sex-matched samples. In the present study, our results support an association between the NRGN gene and schizophrenia. We suggest that the functional haplotype of the NRGN gene, which is associated with NRGN expression, could be related to the pathogenesis of schizophrenia.

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The *p250GAP* Gene Is Associated with Risk for Schizophrenia and Schizotypal Personality Traits

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Abstract

Background: Hypofunction of the glutamate *N*-Methyl-d-aspartate (NMDA) receptor has been implicated in the pathophysiology of schizophrenia. p250GAP is a brain-enriched NMDA receptor-interacting RhoGAP. p250GAP is involved in spine morphology, and spine morphology has been shown to be altered in the post-mortem brains of patients with schizophrenia. Schizotypal personality disorder has a strong familial relationship with schizophrenia. Several susceptibility genes for schizophrenia have been related to schizotypal traits.

Methods: We first investigated the association of eight linkage disequilibrium-tagging single-nucleotide polymorphisms (SNPs) that cover the *p250GAP* gene with schizophrenia in a Japanese sample of 431 schizophrenia patients and 572 controls. We then investigated the impact of the risk genetic variant in the *p250GAP* gene on schizotypal personality traits in 180 healthy subjects using the Schizotypal Personality Questionnaire.

Results: We found a significant difference in genotype frequency between the patients and the controls in rs2298599 ($\chi^2 = 17.6$, $p = 0.00015$). The minor A/A genotype frequency of rs2298599 was higher in the patients (18%) than in the controls (9%) ($\chi^2 = 15.5$, $p = 0.000083$). Moreover, we found that subjects with the rs2298599 risk A/A genotype, compared with G allele carriers, had higher scores of schizotypal traits ($F_{1,178} = 4.08$, $p = 0.045$), particularly the interpersonal factor ($F_{1,178} = 5.85$, $p = 0.017$).

Discussion: These results suggest that a genetic variation in the *p250GAP* gene might increase susceptibility not only for schizophrenia but also for schizotypal personality traits. We concluded that the *p250GAP* gene might be a new candidate gene for susceptibility to schizophrenia.

Introduction

Schizophrenia is a common and complex psychiatric disease. The lifetime morbidity rate is 0.5–1.0% across distinct populations. Family, twin and adoption studies of schizophrenia have indicated that there are strong genetic factors and have estimated the rate of schizophrenia heritability at 80% [1,2]. Although genes implicated in the pathogenesis of schizophrenia have been found using several approaches, such as through association studies of candidate genes, genome-wide association studies (GWAS), copy number variation (CNV) studies and pedigree studies [3,4], the exact genetic factors of this complex disease remain to be explained.

Hypofunction of the glutamate *N*-Methyl-d-aspartate (NMDA) receptor is strongly implicated in the pathophysiology of schizophrenia. NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, mimic symptoms of the disorder in humans and exacerbate symptoms in patients with schizophrenia [5]. These NMDA receptor antagonists induce schizophrenia-like symptoms in humans. Preclinically, they have been shown to

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induce similar symptoms and to induce neural circuitry changes reminiscent of schizophrenia [6]. The ability of these NMDA receptor antagonists to induce a schizophrenia-like phenotype supports the concept that schizophrenia may be the result of reduced or abnormal functioning of NMDA receptors. Altered NMDA receptor binding density in several brain regions, such as in the anterior cingulate cortex, has been reported in schizophrenia [7,8]. The NR2 subunits of the NMDA receptor are spatially and developmentally regulated, and they provide an important level of receptor regulation [9,10]. NR2A and NR2B are the predominant subunits in the cortex, striatum and hippocampus [11,12,13]. In particular, these three areas are closely associated with the pathology of schizophrenia and with the neural circuits within and between these regions [14]. In patients with schizophrenia, alterations have been observed in the NR2 subunit mRNA and protein in the prefrontal cortex, including a reduction in NR2A mRNA and NR2B protein levels [15,16]. Additionally, the NR2B subunit mRNA levels were increased in the hippocampus [17]. Therefore, different expression of NR2 subunits could play an important role in the pathophysiology of schizophrenia.

The NMDA receptor regulates activity-dependent spine morphological plasticity by modulating the actin cytoskeleton [18]. As the key regulators of actin cytoskeleton dynamics, the Rho family of GTPases, including RhoA, Cdc42, and Rac1 and their regulators, play an important role in NMDA receptor-mediated spine morphogenesis [18,19]. In our previous study, we identified the *p250GAP* gene (also known as *p200RHOGAP*, *GRIT*, *KIAA0712*, *RICS*, or *ARHGAP32*: OMIM 608541) as a novel NMDA receptor-interacting RhoGAP [20,21,22,23]. This gene spans approximately 56.17 kb of the genomic DNA and is located on chromosome 11q24.3. p250GAP is highly enriched in the central nervous system, is concentrated in the post-synaptic densities in neurons and is co-localized with the NR2B subunit of the NMDA receptor [20]. Knockdown of p250GAP increased spine width and elevated the endogenous RhoA activity in primary hippocampal neurons, suggesting that p250GAP regulates spine morphogenesis through its RhoGAP activity for RhoA [24]. Importantly, p250GAP activity and localization within neurons are regulated by NMDA receptor activity [20,24], suggesting that p250GAP, together with the NMDA receptor, regulates NMDA receptor-mediated spine morphogenesis. Given that neuropathological studies of schizophrenia have shown alterations in spine morphology [25,26], we hypothesized that the *p250GAP* gene may be related to the pathophysiology of schizophrenia. In this study, we investigated the association between the *p250GAP* gene and schizophrenia in a Japanese population using a gene-based approach.

Schizotypal personality disorder (SPD) is one of the schizophrenia spectrum disorders and is characterized by social avoidance, ideas of reference, vagueness, magical thinking, odd speech, illusions and paranoid ideation. The lifetime prevalence of SPD has been estimated at 3.9% [27], making it one of the more common psychiatric disorders. The prevalence rate of SPD in relatives of individuals with schizophrenia (6.9%) was higher than the prevalence rates found either in relatives of individuals with other psychiatric disorders or in mentally healthy subjects [28]. Twin studies have estimated that the heritability of the latent liability to SPD is 61–72% [29,30]. Premorbid SPD is related to the development of schizophrenia [31]. These findings suggest that SPD shares common genetic influences with schizophrenia. The traits of SPD were incorporated in the SPD criteria in the *Diagnostic and Statistical Manual of Mental Disorders*, third edition (DSM-III), and the traits are listed in the DSM-IV-TR on Axis II. These traits can be identified using a well-validated questionnaire, such as the Schizotypal Personality Questionnaire (SPQ) [32]. The heritability

rates of three schizotypal trait factors, cognitive/perceptual, interpersonal and disorganization, have been estimated at 40 to 60% [33,34]. We recently demonstrated that a genome-wide genetic variant for schizophrenia in the *ZNF804A* gene was associated with schizotypal personality traits [35]. Additionally, we investigated whether a genetic variant in the *p250GAP* gene was associated with schizotypal personality traits in healthy subjects.

Materials and Methods

Ethics statement

Written informed consent was obtained from all subjects after the procedures had been fully explained. This study was performed in accordance with the World Medical Association's Declaration of Helsinki and approved by the Osaka University Research Ethics Committee.

Subjects

The subjects of our genetic association study were 431 patients with schizophrenia (48.7% male (210 males, 221 females), mean age ± SD was 49.7±15.4 years) and 572 healthy controls (46.7% male (267 males, 305 females), mean age ± SD was 61.9±20.4 years). The sex ratio did not differ significantly between the groups ($\chi^2 = 0.41$, $p = 0.52$), but the mean age was significantly different ($z = -11.49$, $p < 0.001$). The subjects were all biologically unrelated and were Japanese. The subjects were recruited from both outpatient and inpatient units at Osaka University Hospital and other psychiatric hospitals. Each patient with schizophrenia had been diagnosed by at least two trained psychiatrists by unstructured clinical interviews, according to the criteria of the DSM-IV. When the diagnosis of the two trained psychiatrists was discordant, they discussed the diagnosis. When the diagnostic disputes were resolved and the patient was diagnosed as schizophrenic, we included the patient. When the diagnostic disputes were not resolved by discussion or the patient was not diagnosed as schizophrenia, we excluded the patient. Controls were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals with current or past contact with psychiatric services, with experience with psychiatric medications or who were not Japanese. We did not assess the controls for their family history of mental disorders, such as schizophrenia, bipolar disorder, or major depressive disorder. The ethnicity was determined by self-report and was not confirmed by genetic analyses.

Data for the schizotypal personality trait analysis were available for 180 healthy subjects [48.3% male (87/93), mean age ± SD: 36.5±11.5 years]. The subjects were included in the genetic association analysis. The subjects included in the analysis met additional criteria. Psychiatrically, medically and neurologically healthy controls were evaluated using the Structured Clinical Interview for DSM-IV-Non-Patient Edition (SCID-I/NP) to exclude individuals who had received psychiatric medications or who had first- or second-degree relatives with psychiatric disorders. Additionally, subjects were excluded from this study if they had neurological or medical conditions that could have potentially affected their central nervous system, such as atypical headaches, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active cancer, cerebrovascular disease, epilepsy, seizures, substance-related disorders or mental retardation.

SNP selection, genotyping and genomic sequencing

This study was designed to examine the association between the *p250GAP* gene and schizophrenia by tagging single-nucleotide

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polymorphisms (SNPs) in the *p250GAP* gene and its flanking regions (±5 kb). Of the 31 SNPs in the *p250GAP* gene and flanking regions, we selected eight tagging SNPs using the TAGGER algorithm (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger) with the criteria of r^2 greater than 0.5 in 'pairwise tagging only' mode and a minor allele frequency (MAF) greater than 5%. The selection was implemented in Haploview 4.2 using HapMap data release 24/PhaseII Nov 08, on NCBI B36 assembly, dbSNP b126 (Japanese in Tokyo (JPT), Chr 11: 128,338,052..128,404,222) (Table S1). The eight tagging SNPs were rs493172, rs10893947, rs2276027, rs3796668, rs581258, rs3740829, rs546239 and rs2298599. The markers are shown in Table 1; the orientation and the alleles are reported on the genomic minus strand. The positions of the eight SNPs analyzed in the present study and the LD relationships between the SNPs in a HapMap JPT population are shown in Figure 1. Venous blood was collected from the subjects. Genomic DNA was extracted from the whole blood using standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems; Foster City, California, USA) as previously described [36,37]. Detailed information on the PCR conditions is available upon request. Genotyping call rates were 99.3% (rs493172), 98.9% (rs10893947), 99.1% (rs2276027), 99.7% (rs3796668), 98.4% (rs581258), 99.2% (rs3740829), 98.5% (rs546239) and 99.3% (rs2298599). No deviations from the Hardy-Weinberg equilibrium (HWE) in the examined SNPs were detected ($p > 0.05$). Additionally, with 48 subjects with schizophrenia, we confirmed a SNP significantly associated with schizophrenia, genotyped by the TaqMan method, using direct DNA sequencing. These subjects were included in the genetic association analysis. The genomic regions were amplified by PCR using a pair of primers for rs2298599, 5′- AAGTCAGCCCA-GACTCTCCA -3′ and 5′- GAGGGAGGAAGGGATTTTTG -3′. PCR for each sample was carried out in a total volume of 40 μl using a Gene Amp® PCR System 9700 (Applied Biosystems, CA, U.S.A). The PCR cycling conditions were 94°C for 10 minutes, 30 cycles at 94°C for 1 minute, 60°C for one minute and 72°C for 1 minute, followed by an incubation at 72°C for 10 minutes. The PCR products were purified using a QIA quick® PCR Purification Kit (QIAGEN, CA, USA), and the purification products were sequenced using a Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Cycle sequencing conditions were 96°C for 2 minutes, 25 cycles of 96°C for 20 seconds, 50°C for 30 seconds and 60°C for 2 minutes, using a Gene Amp® PCR System 9700. The PCR products from the cycle sequencing were purified using a Big Dye® XTerminator™ Purification Kit (Applied Biosystems, CA, U.S.A.), and they were sequenced using an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, CA, U.S.A.). The sequencing was checked with SEQUENCHER ver. 4.7 (Gene Codes, U.S.A.).

Schizotypal personality trait analysis

To assess schizotypal personality traits, a full Japanese version of the SPQ was administered to healthy subjects [38,39]. The SPQ is a 74-item self-report questionnaire with a "yes/no" response format [40]. All items answered "yes" were scored 1. The SPQ measures nine subscales of specific schizotypal features, which are ideas of reference, odd beliefs/magical thinking, unusual perceptual experiences, suspiciousness/paranoid ideation, social anxiety, no close friends, constricted affect, eccentric/odd behavior and odd speech. The total SPQ score was obtained by summing the scores from all of the items. The three schizotypal trait factors, cognitive/perceptual, interpersonal and disorganization, were derived by summing the related subscale raw scores according to the three-factor model of Raine and colleagues [32]. Full-scale IQ was assessed using the Wechsler Adult Intelligence Scale, Revised or Third edition.

Statistical analysis

Differences in clinical characteristics between the patients and the controls or between the genotype groups were analyzed using the χ^2 test for categorical variables and the Mann-Whitney U-test for continuous variables, using the PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). We performed power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS [41]). The power estimates were based on the allele frequency of 0.35 (rs2298599) in the controls and an alpha level of 0.05. Power was calculated under a prevalence of 0.01 using a multiplicative model that assumed varying degrees of the odds ratio (OR). Statistical analyses for the genetic associations were performed using the SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). Deviation from the HWE was tested using χ^2 tests for goodness of

Table 1. Genotypic and allelic distributions for SNPs in the *p250GAP* between patients with schizophrenia and controls.

Marker				SCZ (n=431)			CON (n=572)			Genotypic	SCZ	CON	Allelic	OR
SNP IDs	Position[a]	M/m	gene	M/M	M/m	m/m	M/M	M/m	m/m	p (χ^2)	MAF		p (χ^2)	(95% CI)
rs493172	128388089	C/G	intron1	346	77	3	451	116	3	0.63 (0.9)	0.10	0.11	0.49 (0.5)	0.90 (0.67–1.21)
rs10893947	128375634	G/A	intron1	122	217	88	177	294	94	0.25 (2.8)	0.46	0.43	0.14 (2.2)	1.15 (0.96–1.37)
rs2276027	128355514	T/C	intron8	241	158	27	303	229	36	0.57 (1.1)	0.25	0.27	0.42 (0.7)	0.92 (0.75–1.13)
rs3796668	128349062	A/C	intron11	186	182	62	206	292	72	**0.020 (7.8)**	0.36	0.38	0.22 (1.5)	0.89 (0.74–1.07)
rs581258	128348083	A/G	exon12	293	125	8	373	171	17	0.46 (1.6)	0.17	0.18	0.32 (1.0)	0.89 (0.70–1.12)
rs3740829	128344366	A/G	exon13	375	50	2	513	54	1	0.37 (2.0)	0.06	0.05	0.18 (1.8)	1.30 (0.89–1.91)
rs546239	128340968	A/G	3′	325	91	9	402	149	12	0.18 (3.4)	0.13	0.15	0.11 (2.6)	0.81 (0.63–1.05)
rs2298599	128340162	G/A	3′	167	184	76	219	296	53	**0.00015 (17.6)**	0.39	0.35	0.07 (3.3)	1.18 (0.99–1.42)

SCZ: patients with schizophrenia, CON: controls, M: major allele, m: minor allele, MAF: minor allele frequency, OR: odds ratio, 95%CI: 95% confidence interval.
[a] db SNP build 129.
All of the alleles are represented according to the minus strand DNA sequence. Numbers of genotypes were represented as genotype counts. *P* values<0.05 are in boldface and underlined.
doi:10.1371/journal.pone.0035696.t001

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