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Ⅳ.研究成果の刊行物・別刷

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No association between the ryanodine receptor 3 gene and autism in a Japanese population

(Running title: No association between RyR3 and autism)

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Abstract

Aim: Autism is a neurodevelopmental disorder with a complex genetic etiology. Chromosome 15q11-q14 has been proposed to harbor a gene for autism susceptibility because deletion of the region leads to Prader-Willi syndrome or Angelman syndrome, having phenotypic overlap with autism. Here we studied the association between autism and the ryanodine receptor 3 *(RyR3)* gene, which is located in the region. This is the first study which investigated the association, to our knowledge.

Methods: We genotyped 14 tag single nucleotide polymorphisms (SNPs) in 166 Japanese patients with autism and 375 controls.

Results: No significant difference was observed between the patients and controls in allelic frequencies or genotypic distributions of the 14 SNPs. Analysis after confining the subjects to males showed similar results.

Conclusions: The present study provides no positive evidence for the association between the *RyR3* gene and autism in the Japanese population.

Key words: autism; chromosome 15; ryanodine receptor; association study; single nucleotide polymorphism (SNP)

Introduction

Autism is a developmental disorder characterized by three areas of abnormality: impairment in social interaction, impairment in communication, and restricted and stereotyped pattern of interest or behavior. Impairment in all three areas is observed before age three years and disrupted growth of the brain, with unknown mechanism, is implicated in the etiology of autism. Twin and family studies have indicated a robust role of genetic factors in the development of autism, while few susceptibility genes have been elucidated[1]. Chromosome 15q11-q14 is a region highly susceptible to clinically important genomic rearrangements, including interstitial deletions, duplications, triplications, and the generation of supernumerary marker chromosomes (SMCs), called "idic" or "inverted duplication"[2]. Deletions of the region lead to Prader-Willi syndrome and Angelman syndrome depending on the deleted chromosome's parent of origin[3]. Both syndromes have phenotypic overlap with autism, therefore, chromosome 15q11-q14 has been proposed to harbor a gene for autism susceptibility[4, 5].

The ryanodine receptor 3 *(RyR3)* gene has been mapped to chromosome 15q14-q15[6]. It is an isoform of RyRs and expressed at high levels in caudate nucleus, amygdala, and hippocampus in the central nervous system[7]. An animal study showed that deletion of the *RyR3* changes hippocampal synaptic plasticity, specifically on the adaptation of acquired memory in response to external changes or stimuli, without affecting hippocampal morphology, basal synaptic transmission or presynaptic function[8]. Calcium-induced calcium release from a ryanodine-sensitive Ca^{2+} store may be required for the induction of contextual learning[9]. Restricted and stereotyped pattern of interest or behavior without adjusting external changes or stimuli is one of the three characteristics of autism. On the basis of the location and function of the gene, *RyR3* may be a candidate for autism susceptibility. To our knowledge, however, no study has investigated the genetic association between *RyR3* and autism to date. Here we investigated the association between the *RyR3* gene and autism in a Japanese population.

Methods

Subjects

In this study, Japanese patients and control subjects around Tokyo, Japan, were recruited: 166 unrelated patients with autistic disorder (147 males and 19 females; age, 19.9 ± 9.8 years, mean ± SD) and 375 unrelated healthy volunteers (127 males and 248 females; age: 36.0 ± 11.7 years). Two experienced child-psychiatrists independently conducted a semi-structured behavior-observation of them and interview of all patients and their parents, and made final diagnosis according to the ICD-10 DCR and DSM-IV. The cases which fulfilled both ICD-10 and DSM-IV criteria were included in the present study and followed up for six months. We excluded the cases which were found not to fulfill both criteria within the period. At the interview, the Child Behavior Questionnaire Revised[10] was used to assist the evaluation of the autism-specific behaviors and symptoms. In order to exclude other genetic syndromes, we performed standard karyotyping and fragile X testing for the trinucleotide repeat expansion in the *FMR-1* gene[11]. IQ levels were > 70 in 12 patients, 50 – 70 in 33 patients, 35 – 50 in 30 patients, and < 35 in 37 patients. The levels were evaluated mainly using a Japanese version of the Binet test. Thirty-nine patients were unable to take the IQ test due to their communication disorders or disability to understand the questions. Data was not available in other 15 patients. The objective of the present study was clearly explained, and written informed consent was obtained from all parents. The consent was also obtained from the patients when they were able to follow the explanation (approximately mental age is > 6 years old). Controls were mainly recruited from the hospital and facility staff. All controls received a short interview by one of the authors to confirm that they had no history of major mental illness. The study was approved by the Ethical Committee of the Faculty of Medicine, the University of Tokyo.

Genotyping

Genomic DNA was extracted from leukocytes by using the standard phenol-chloroform method. We genotyped 14 single nucleotide polymorphisms (SNPs) as detailed in Table 1. All length of the gene was covered by selecting tag SNPs by using SNP Wizard module (SNP Tag Selection, pair-wise r^2 = 99%) of SNPbrowser™ software (Applied Biosystems, CA), in which haplotype blocks are estimated depending on the data from

the International HapMap project[12] or Applied Biosystems. All SNPs were analyzed by TaqMan PCR method using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Statistical method

The chi-square test was used to compare the allele or genotype frequencies between the patients and controls. Lewontin's D' was used to analyze pairwise linkage disequilibrium (LD)[13]. Haplotype block analysis was conducted in the Gabriel method as well as the Four Gamete method[14, 15]. Haploview 3.32[16] was used to conduct LD and haplotype block analyses. Statistical power was calculated by using Genetic Power Calculator[17].

Results

Table 1 shows allele and genotype frequencies of the 14 SNPs compared between the patients and controls. The distributions of all 14 SNPs follow the Hardy-Weinberg equilibrium both in the patients and controls. No significant difference was observed in allelic frequencies or genotypic distributions of the 14 SNPs between the patients and controls. Analysis after confining the subjects to males showed no significant difference (data not shown).

The strength of LD denoted as D' between pairs of SNPs is shown in Figure 1. No haplotype block was suggested by the Gabriel or Four Gamete method of haplotype block analysis[14, 15], which had been expected considering definition of tag SNPs. The LD analysis in males showed similar results (data not shown).

Discussion

In the present study, we investigated the possible association between the *RyR3* gene and autism by analyzing 14 tag SNPs. No significant difference was observed between the patients and controls in allelic frequencies or genotypic distributions of the 14 SNPs. Analysis after confining the subjects to males showed similar results. Thus, the present study provides no positive evidence for the association between the *RyR3* gene and autism in the Japanese population.

Statistical power of the present study is 0.76 (α = 0.05) when assuming that the prevalence of autism is 0.21% in the Japanese population[18], genotypic relative risk is 1.8 (dominant model), and risk allele frequency is 0.1. Thus, our results might have adequate statistical power to detect the effect of the gene with odds ratios of approximately 1.8 or more at nominal p-value of 0.05, although the power is reduced when multiple testing is considered and also, smaller effects might not be detected in the present sample. Caution may be needed for the controls in the present study because they were not age-matched to the patients. However, this may not be likely to significantly affect the result, considering no major effect of environmental factors in autism[19]. Imbalance in sex ratio between the patients and controls may be overcome by analysis confining the subjects to males considering its higher prevalence in males than in females. A wide range of IQ levels in the patients may be another limitation in the present study. Heterogeneity in the present subjects might affect the results.

In conclusion, no evidence was obtained for a role of the *RyR3* gene in the development of autism. However, rare variant or epigenetic factor was not investigated in the present study. Further investigation of the region including these factors may be recommended.

Figure legend

Figure 1. The strength of LD between pairs of SNPs in all subjects. The strength of LD is denoted as D' (100 × exact D' value).

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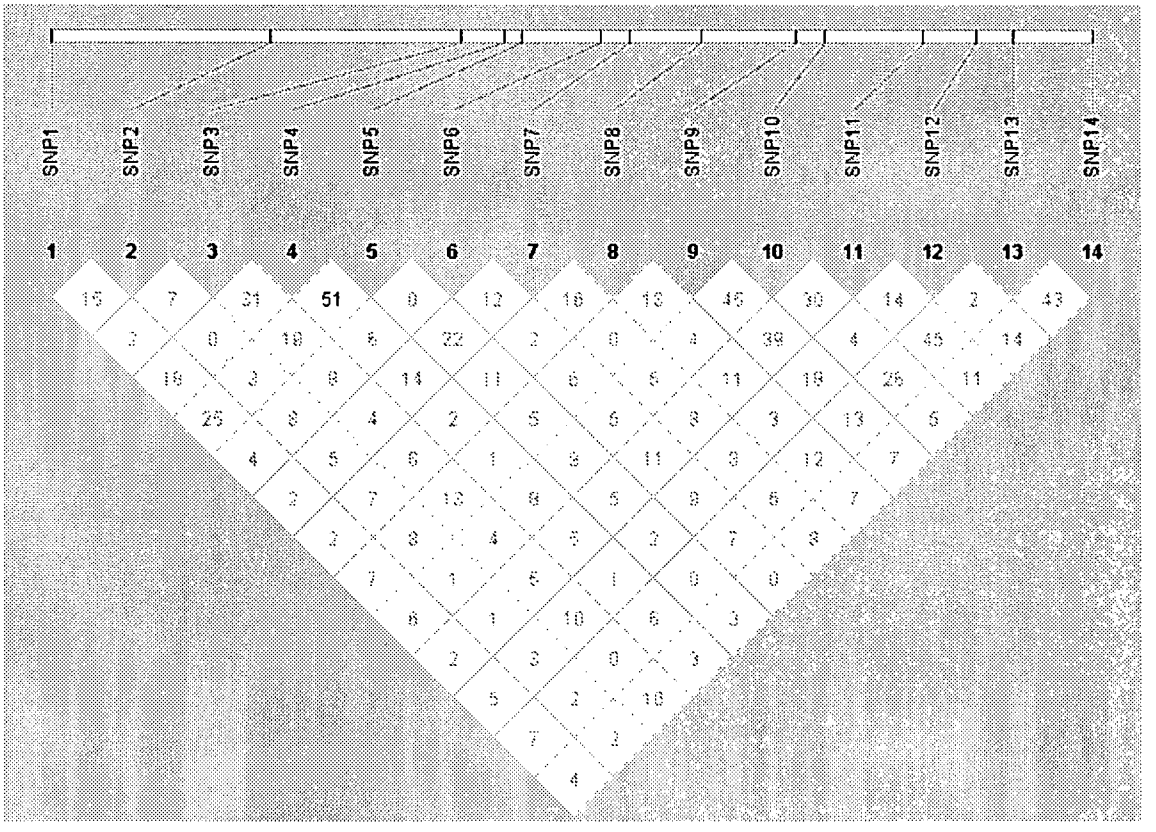
Table 1. Allele and genotype frequencies of 14 SNPs in the *RYR3* gene

SNPs	db SNP ID	Location	Alleles (Major/Minor)	Minor allele frequency		Genotype frequencies**		Chromosome position (bp)
				Autism*	Control*	Autism	Control	
SNP1	rs2676052	intron 1	C/T	0.37 (166)	0.35 (365)	0.38/0.50/0.12	0.43/0.45/0.12	10483443
SNP2	rs2596175	intron 2	T/A	0.44 (166)	0.46 (371)	0.31/0.50/0.19	0.29/0.50/0.21	10581450
SNP3	rs1435118	intron 12	A/G	0.39 (166)	0.44 (370)	0.39/0.45/0.16	0.31/0.50/0.19	10667021
SNP4	rs640152	intron 16	G/A	0.27 (166)	0.26 (372)	0.55/0.35/0.10	0.55/0.39/0.06	10686057
SNP5	rs2467565	intron 16	A/G	0.26 (166)	0.21 (371)	0.55/0.39/0.06	0.61/0.35/0.04	10694156
SNP6	rs1495280	intron 25	T/C	0.35 (166)	0.33 (368)	0.43/0.44/0.13	0.44/0.45/0.11	10729162
SNP7	rs937303	intron 30	A/G	0.40 (166)	0.39 (375)	0.36/0.48/0.16	0.37/0.47/0.16	10742164
SNP8	rs12907278	intron 38	G/A	0.48 (166)	0.43 (374)	0.30/0.45/0.25	0.31/0.51/0.18	10774578
SNP9	rs2293027	exon 44	G/A	0.20 (166)	0.22 (371)	0.63/0.35/0.02	0.61/0.34/0.05	10816402
SNP10	rs2288606	intron 49	C/A	0.27 (166)	0.27 (373)	0.54/0.37/0.09	0.53/0.39/0.08	10829532
SNP11	rs12916967	intron 62	A/G	0.44 (164)	0.43 (373)	0.32/0.48/0.20	0.31/0.53/0.16	10873068
SNP12	rs11072673	intron 67	G/T	0.42 (164)	0.45 (372)	0.32/0.52/0.16	0.31/0.47/0.22	10897620
SNP13	rs1036005	intron 75	T/C	0.25 (164)	0.25 (373)	0.55/0.41/0.04	0.59/0.33/0.08	10914237
SNP14	rs713202	intron 95	C/T	0.26 (165)	0.31 (349)	0.53/0.42/0.05	0.48/0.43/0.09	10949706

* Number of genotyped individuals for each SNP is given in parenthesis.
** Described as major homo/hetero/minor homo.



Association study of the commonly recognized breakpoints in chromosome 15q11–q13 in Japanese autistic patients

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Objective **Chromosome 15q11–q13 has been proposed to harbor a gene for autism susceptibility because deletions of the region lead to Prader–Willi syndrome and Angelman syndrome, whose phenotypes overlap with autism. These deletions generally occur with the use of three commonly recognized breakpoints (BP1, BP2, and BP3); therefore, it may be possible that genes located in the breakpoints are impaired and contribute to autism susceptibility. No study, however, has investigated the genetic association between the breakpoints and autism, to our knowledge. Here, we investigated the association between the common breakpoints of chromosome 15q11–q13 and autism in a Japanese population.**

Methods **We genotyped 12 single nucleotide polymorphisms (SNPs) in 166 patients with autistic disorder and 415 healthy controls. The SNPs are located in two additional distal breakpoints (BP4 and BP5), involved in duplications and triplications of the region, as well as in BP1 and BP3.**

Results **No significant difference was observed between the controls and patients in allelic frequencies or genotypic distributions of the 12 SNPs. In the analyses of the suggested five haplotypes, no significant difference between the controls and patients was observed in the distributions of any estimated haplotypes. When confining the patients to only males, a difference was observed in a two-marker haplotype in BP3 between the controls and patients (global permutation *P* value=0.006), although the statistical level became insignificant after correction for multiple testing.**

Conclusion **This study provides no positive evidence of the association between the common breakpoints of chromosome 15q11–q13 and autism in the Japanese population. *Psychiatr Genet* 00:000–000 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: Angelman syndrome, amyloid precursor protein-binding protein A2, autism, breakpoint, chromosome 15, Prader–Willi syndrome

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Introduction

Autism is a developmental disorder characterized by three areas of abnormality: impairment in social interaction, impairment in communication, and restricted and stereotyped pattern of interest or behavior. Impairment in all three areas is observed before the age of 3 years and disrupted growth of the brain, with unknown mechanism, is implicated in the etiology of autism. Twin and family studies have indicated a robust role of genetic factors in the development of autism, although few susceptibility genes have been elucidated (Freitag, 2007). Chromosome 15q11–q13 is a region highly susceptible to clinically important genomic rearrangements, including interstitial deletions, duplications, triplications, and the generation of supernumerary marker chromosomes (SMCs) called 'idic' or 'inverted duplication' (Wang *et al.*, 2004). Deletions of the region lead to Prader–Willi syndrome and Angelman syndrome depending on the deleted chromosome's parent of origin (Knoll *et al.*, 1989). These deletions generally occur with the use of three commonly recognized breakpoints (BP1, BP2, and BP3), whereas duplications and triplications have been described that involve two additional distal breakpoints (BP4 and BP5) (Wang *et al.*, 2004). Both syndromes have phenotypic overlap with autism; therefore, chromosome 15q11–q13 has been proposed to harbor a gene for autism susceptibility (Peters *et al.*, 2004; Veltman *et al.*, 2004). Several studies have assessed genes located on the region, such as

GABRB3, *GABRG3*, *ATP10C*, and *UBE3A*, in autism, although the results were quite controversial (Freitag, 2007). To our knowledge, no study has investigated the genetic association between the breakpoints and autism to date. It may be possible that genes located in the breakpoints are impaired and contribute to autism susceptibility. In this study, we investigated the association between the common breakpoints of chromosome 15q11–q13 and autism in a Japanese population.

Patients and methods

In this study, Japanese patients and controls around Tokyo, Japan, were recruited: 166 unrelated patients with autistic disorder (147 males and 19 females; age: 19.9 ± 9.8 years, mean ± SD) and 415 unrelated healthy volunteers (138 males and 277 females; age: 36.0 ± 11.5 years). The patients were recruited in clinical settings from the outpatient clinics of the departments of psychiatry, University of Tokyo Hospital and Tokai University Hospital, and seven day care facilities for patients with developmental disorders. All the hospitals and facilities were located around Tokyo. Controls were mainly recruited from among the hospital and facility staff. All the controls resided in the same area (Kanto District or around Tokyo) as the patients. Two experienced child-psychiatrists independently conducted a semistructured behavior-observation of them and an interview of all the patients and their parents, and made a final diagnosis according to the ICD-10 DCR and Diagnostic and Statistical Manual Of Mental Disorder-IV. The cases which fulfilled both ICD-10 and Diagnostic and Statistical Manual Of Mental Disorder-IV criteria were included in this study and followed up for 6 months. We excluded the cases that were found not to fulfill both criteria within the period. At the interview, the Child Behavior Questionnaire-Revised (Izutsu *et al.*, 2001) was used to assist the evaluation of the autism-specific behaviors and symptoms. To exclude other genetic syndromes or neurological diseases, tests were administered to the patients that included a full exploration of medical and family history, physical, and neurological examinations such as brain imaging, EEG, urinalysis, standard karyotyping, and fragile X testing for the trinucleotide repeat expansion in the *FMR-1* gene (Chong *et al.*, 1994). IQ levels were > 70 in 12 patients, 50–70 in 33 patients, 35–50 in 30 patients, and < 35 in 37 patients. The levels were evaluated mainly using a Japanese version of the Binet test. Thirty-nine patients were unable to take the IQ test owing to their communication disorders or disability to understand the questions. Data were not available in case of 15 patients. The objective of this study was clearly explained, and written informed consent was obtained from all the parents. The consent was also obtained from the patients when they were able to follow the explanation. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Tokyo.

Genomic DNA was extracted from leukocytes by using the standard phenol-chloroform method. We genotyped 12 single nucleotide polymorphisms (SNPs) as detailed in Table 1. SNPs with minor allele frequencies (MAF) > 30% were selected by using the SNP Wizard module of SNPbrowser software (Applied Biosystems, California, USA), from the region of BP1, BP3, BP4, and BP5. No SNP with MAF > 30% was available in the region of BP2. The SNPs were analyzed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The χ^2 test was used to compare the SNP frequencies between the controls and patients. Lewontin's D′ was used to analyze pairwise linkage disequilibrium (LD) (Lewontin, 1964). Haplotype block analysis was conducted using the Gabriel method as well as the Four Gamete method (Gabriel *et al.*, 2002; Wang *et al.*, 2002). Haplotypes of the SNPs and their frequencies were estimated by the maximum-likelihood method with an expectation-maximization algorithm (Excoffier and Slatkin, 1995). Permutation *P* values were calculated in comparison of haplotype frequencies between the patients and controls (Fallin *et al.*, 2001). The SNPAlyze 5.1 Standard software (Dynacom, Yokohama, Japan) was used to conduct LD, haplotype block, and haplotype analyses.

Results

Table 1 shows allelic frequencies of the 12 SNPs compared between the controls and patients. The distributions of all 12 SNPs follow the Hardy–Weinberg equilibrium both in the controls and patients. No significant difference was observed in the allelic frequencies or the genotypic distributions of the 12 SNPs between the controls and patients. An analysis after confining the patients to only males showed no significant difference (data not shown).

The strength of LD denoted as D′ between pairs of SNPs in each breakpoint is shown in Table 2. In BP3, two haplotype blocks (A-B-C and D-E) were suggested by the Gabriel and Four Gamete methods of haplotype block analyses; in BP5, only one haplotype block (B-C) was suggested (Gabriel *et al.*, 2002; Wang *et al.*, 2002). We therefore analyzed five haplotypes: BP1-A-B, BP3-A-B-C, BP3-D-E, BP4-A-B, and BP5-B-C. No significant difference between the controls and patients was observed in the distributions of any estimated haplotypes in those five haplotypes. When confining the patients to only males, however, there was a significant difference in the distribution of haplotype BP3-D-E between the controls and patients (global permutation *P* value = 0.006; Table 3). The frequency of the estimated haplotype 'C-C' is significantly larger in the patients than in the

Table 1 Allelic frequencies of 12 SNPs

SNPs	db SNP ID	Location	Alleles (Major/minor)	Minor allele frequency			Chromosome Position (bp)
				Autism[a]	Control[a]	*P* value	
BP1-A	rs35571762	*TUBGCP5*	G/A	0.46 (161)	0.46 (412)	0.93	1525004
BP1-B	rs34146883	*TUBGCP5*	T/C	0.48 (163)	0.47 (409)	0.75	1547452
BP3-A	rs1873279	*APBA2*	G/A	0.45 (164)	0.48 (413)	0.46	6882863
BP3-B	rs12443112	*APBA2*	G/A	0.41 (164)	0.43 (412)	0.54	6907289
BP3-C	rs12437939	*APBA2*	G/A	0.42 (163)	0.43 (415)	0.65	6908621
BP3-D	rs4779758	*APBA2*	C/T	0.31 (165)	0.33 (413)	0.36	7021597
BP3-E	rs1565401	*APBA2*	G/C	0.33 (166)	0.34 (412)	0.83	7034006
BP4-A	rs1004818	–	G/C	0.46 (166)	0.47 (413)	0.80	7940432
BP4-B	rs7173960	–	T/C	0.49 (166)	0.49 (414)	0.96	7943846
BP5-A	rs1406387	*SCG5*	C/T	0.41 (166)	0.47 (410)	0.10	9760540
BP5-B	rs12438604	*SCG5*	A/C	0.39 (166)	0.42 (413)	0.38	9774845
BP5-C	rs11638903	*SCG5*	A/T	0.27 (166)	0.27 (415)	1.00	9781573

APBA2, amyloid precursor protein-binding protein A2; *SCG5*, secretogranin V; *TUBGCP5*, tubulin, gamma complex associated protein 5.
BP, breakpoint; SNP, single nucleotide polymorphism.
[a]Number of genotyped individuals for each SNP is given in parenthesis.

Table 2 The strength of LD between pairs of SNPs in patients and controls

a. BP1	BP1-A	BP1-B			
BP1-A		0.97			
BP1-B	0.99				
b. BP3	BP3-A	BP3-B	BP3-C	BP3-D	BP3-E
BP3-A		0.94	0.94	0.26	0.29
BP3-B	0.99		1.00	0.34	0.35
BP3-C	0.99	1.00		0.36	0.37
BP3-D	0.05	0.12	0.12		1.00
BP3-E	0.05	0.11	0.11	0.99	
c. BP4	BP4-A	BP4-B			
BP4-A		1.00			
BP4-B	0.98				
d. BP5	BP5-A	BP5-B	BP5-C		
BP5-A		0.55	0.48		
BP5-B	0.52		1.00		
BP5-C	0.58	0.98			

*The strength of LD is denoted as D′.
**The values of D′ for patients are shown in the upper diagonal and those for controls are shown in the lower diagonal.
BP, breakpoint; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

Table 3 Estimated haplotype frequencies of the haplotype BP3-D-E

BP3-	D	E	Frequency in all patients*			Frequency in males**		
			Autism	Control	Permutation *P* value	Autism	Control	Permutation *P* value
	C	G	0.666	0.659	0.838	0.667	0.653	0.790
	T	C	0.307	0.333	0.408	0.303	0.347	0.283
	C	C	0.027	0.0086	0.023	0.0306	<0.0001	0.0039

Haplotypes whose frequencies were estimated >1% were described.
BP, breakpoint.
*Global permutaion *P* value=0.045.
**Global permutation *P* value=0.006.

controls (0.0306 vs. <0.0001, respectively, permutation *P* value = 0.0039). No significant difference between the controls and patients was observed in the distributions of the other five haplotypes.

Discussion

In this study, we investigated the possible association between autism and 12 SNPs in the four common breakpoints of the chromosome 15q11–q13 region. No significant difference was observed between the controls and patients in allelic frequencies or genotypic distributions of the 12 SNPs. Permutation tests showed no significant global difference in the estimated haplotype frequencies between the controls and patients. When confining the patients to only males, a significant difference was observed in the haplotype BP3-D-E

between the controls and patients (global permutation P value = 0.006). The statistical level, however, became insignificant after correction for multiple testing for five haplotype analyses in total and male patients. Thus, this study provides no positive evidence of the association between the common breakpoints of chromosome 15q11–q13 and autism in the Japanese population.

SNPs BP3-D and BP3-E are located in the gene encoding the amyloid precursor protein-binding protein A2 (APBA2) (Sutcliffe *et al.*, 2003). APBA2 has also been termed as Mint2, for Munc-interacting protein 2, or X-11β, which functions as a neuronal adaptor protein. Mint2 binds directly to the cytoplasmic tail of neurexins and facilitates synaptic vesicle exocytosis (Biederer and Sudhof, 2000). Although no significant association was observed in the present result, the location and function make *ABPA2* an attractive candidate for the autism susceptibility gene. Denser mapping of the locus around SNPs BP3-D and BP3-E may be worth investigating.

Caution may be needed for the controls in this study because they were not age-matched with the patients. This may, however, be unlikely to significantly affect the result, considering that there is no major effect of environmental factors in autism (Folstein and Rosen-Sheidley, 2001). Imbalance in sex ratio between the patients and controls may be overcome by an analysis confining the patients to only males considering its higher prevalence in males than in females. Another limitation may be diagnostic issues. Neither the ADOS (Autism Diagnostic Observation Schedule) (Lord *et al.*, 1989) nor the ADI-R (Autism Diagnostic Interview-Revised) (Lord *et al.*, 1994) has been available in Japan to date. The existence of bias perhaps cannot be denied in the diagnosis of the present patients, although its effect is less if any at all..

In conclusion, no significant association was observed between the common breakpoints of chromosome 15q11–q13 and autism in the present Japanese patients.

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Association study of the 15q11-q13 maternal expression domain in Japanese autistic patients

(Running head: association between the MED and autism)

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