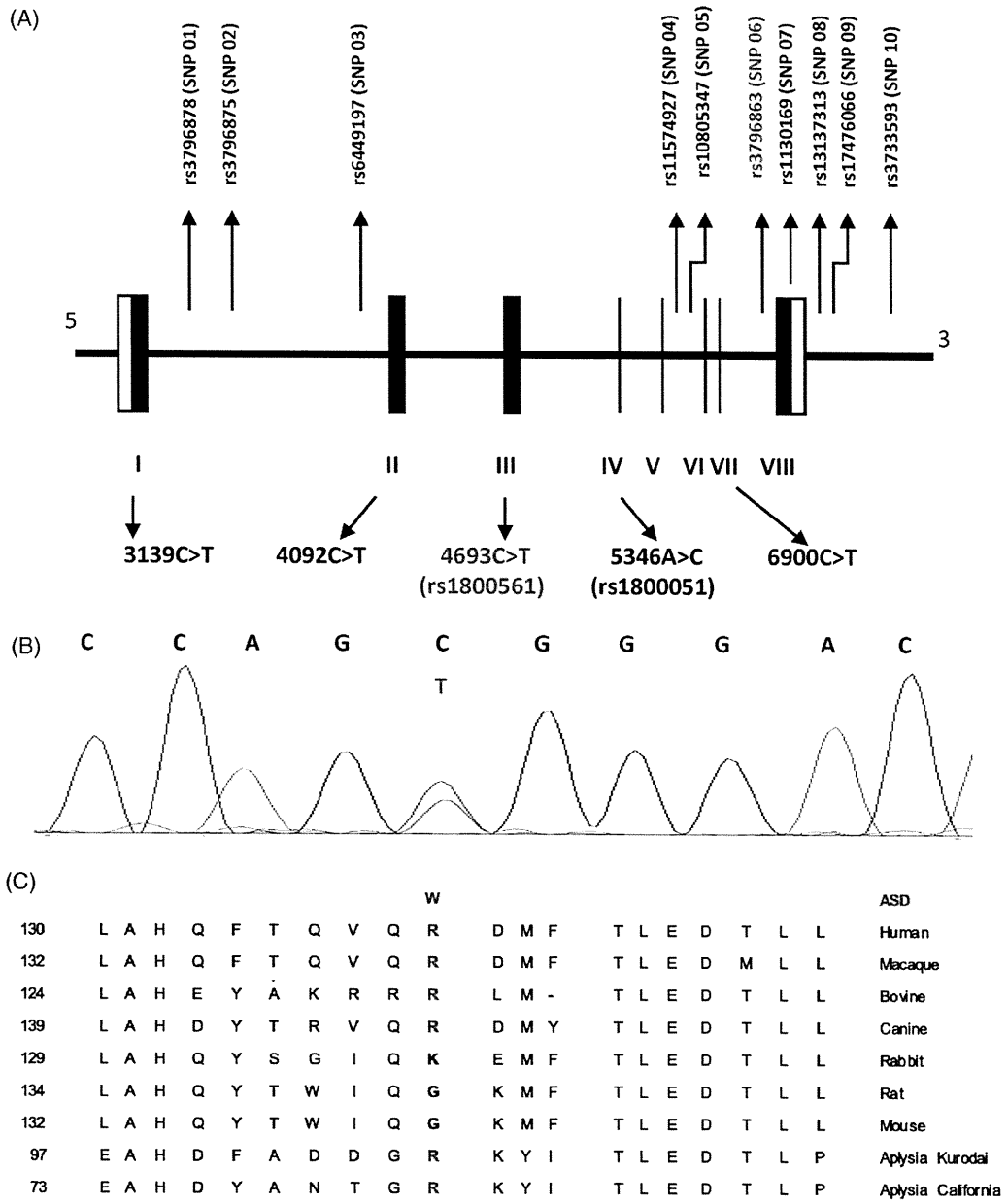

Fig. 2. Genome structure, SNPs, and mutations of *CD38*. (A) Genomic structure of *CD38* and locations of SNPs in introns (upper) and exons (lower). Exons are indicated by boxes, with translated regions in closed boxes and untranslated regions in open boxes. Numbering of the nucleotides starts at the A of ATG and refers to GenBank Accession number D84284. (B) The sequence trace was derived from a DNA sample of a 4693C/T heterozygote. (C) Amino acid sequence of CD38 showing conservation of R at the 140th amino acid among different species, except for rodents and rabbits. Sequences were obtained through the accession numbers NM001775, AY555148, NM175798, AF117714, AF272974, NM013127, NM007646, D30048, and M85206/M37644 for the indicated species.

2.6. Statistics

We analyzed the data using one- or two-way ANOVA, as appropriate. The criterion for significance in all cases was $p < 0.05$. PedCheck program v1.1 (http://watson.hgen.pitt.edu) was used to identify and eliminate all Mendelian inheritance inconsistencies in the trio genotype data. Markers were tested for association by family-based association test (FBAT), using FBAT v2.0.3 (http://www.biostat.harvard.edu/~fbat/).

3. Results

The highest level of *CD38* mRNA expression was detected in the human hypothalamus, and we detected substantial expression in the frontal cortex, amygdala, and cerebellum (data not shown). CD38 immunoreactivity was detected in the hypothalamus of the two Japanese brains (data not shown). In brains of samples from USA (Fig. 1), double immunohistochemical staining revealed high levels of CD38 immunoreactivity in many cells in the paraventricular nucleus of the hypothalamus and showed extensive colabeling with OT (Yamashita et al., 2002), while much lower CD38 expression levels and little or no detectable OT were observed in the insular cortex, which served as a control. These results suggested that CD38 may have an important role in OT release in the human hypothalamus, as in the mice (Jin et al., 2007; Liu et al., 2008). Based on this new information about the human brain, we set out to examine the human *CD38* gene.

Table 4
FBAT analysis of *CD38* SNPs in AGRE trios.

Marker	Allele	252 trios				HFA trios			
		Families[a]	Frequency	Z-Score	p-Value	Families[a]	Frequency	Z-Score	p-Value[b]
rs3796878	A	119	0.163	0.253	0.801	49	0.161	0.640	0.522
	G	119	0.837	−0.253		49	0.839	−0.640	
rs3796875	A	171	0.659	−0.740	0.459	78	0.640	−0.594	0.552
	G	171	0.341	0.740		78	0.360	0.594	
rs6449197	T	79	0.103	−0.647	0.518	30	0.103	−2.058	***0.040***
	C	79	0.897	0.647		30	0.897	2.058	
rs11574927	A	111	0.830	0.947	0.344	55	0.834	1.861	0.063
	G	111	0.170	−0.947		55	0.166	−1.861	
rs10805347	A	171	0.321	−0.614	0.539	71	0.309	−0.663	0.508
	G	171	0.679	0.614		71	0.691	0.663	
rs3796863	A	160	0.277	−1.706	0.088	74	0.270	−2.800	***0.005***
	C	160	0.723	1.706		74	0.730	2.800	
rs1130169	T	195	0.503	1.004	0.315	85	0.517	0.659	0.510
	C	195	0.497	−1.004		85	0.483	−0.659	
rs13137313	A	168	0.742	0.765	0.445	72	0.726	0.426	0.670
	G	168	0.258	−0.765		72	0.274	−0.426	
rs17476066	T	167	0.714	0.697	0.486	69	0.714	0.655	0.513
	C	167	0.286	−0.697		69	0.286	−0.655	
rs3733593	T	178	0.451	0.962	0.336	74	0.428	0.887	0.375
	C	178	0.549	−0.962		74	0.572	−0.887	
p-Value after multimarker testing					0.295				0.053

[a] Informative families.
[b] Significant p-values are indicated in bold italic.

3.1. Intronic SNP analysis in Japanese and U.S. subjects

An association study for 10 intronic SNPs shown in Fig. 2 was first performed in a case–control study in a Japanese population (29 ASD subjects and 315 controls, the first cohort in the Table 1). No significant association with ASD was found for these SNPs (data not shown).

Next, we analyzed U.S. ASD DNA samples (cohort 4). FBAT was performed for the whole set of 252 trios in the AGRE samples. Again, none of the SNPs showed significant associations, except rs3796863 (SNP06) with a tendency toward association ($p = 0.088$; Table 4). Therefore, we further analyzed this SNP for the U.S. HFA subgroup of 104 trios in our AGRE samples (cohort 4). In the FBAT of HFA trios, rs6449197 ($p = 0.040$) and rs3796863 ($p = 0.005$) showed significant associations; a tendency for association ($p = 0.053$) was found after multimarker testing (Table 4).

Unlike the U.S. cases, no association was detected in 188 Japanese HFA trio cases selected from cohort 2 ($p = 0.228$).

One-way ANOVA showed a significant variation in the distribution of ADI-R_C scores (restricted, repetitive, and stereotyped patterns of behavior) between the C/C, C/A, and A/A genotypes of SNP06 of *CD38*, in 252 trios ($p = 0.013$) and HFA trios ($p = 0.0067$) (Fig. 3). Following *post hoc* pairwise comparison with the Bonferroni method, the variations in the distribution of ADI-R_C between the C/C and C/A groups were found to be significant at the 0.05- and 0.01-levels, in the 252 and HFA trios, respectively.

Linkage disequilibrium (LD) analysis identified three haplotype blocks across the *CD38* gene in 104 trios in AGRE samples, with the first block comprising SNPs01-05, the second block comprising SNPs06 and 07, and the third block comprising SNPs08 and 09 (Fig. 4). The results of haplotype transmission disequilibrium test (TDT) for the HFA trios are shown in Table 5. The associations of haplotypes in the three haploblocks were examined based on the LD structure of *CD38*. The first haploblock including SNPs01-05, showed a weak tendency for association ($p = 0.055$; Table 5). The haplotypes GGCAG ($p = 0.022$) and GGTAG ($p = 0.034$) of this block showed significant associations; however, this was not significant by permutation (permutation $p = 0.157$ for GGCAG and permutation $p = 0.271$ for GGTAG). The GGCAG haplotype, with the C allele of SNP03, showed overtransmission (62.61%). Overtransmission (51.58%) of the C allele of SNP03 was also observed in single SNP TDT.

Table 5
Haplotype associations of SNPs belonging to the three LD blocks of *CD38* in HFA trios.

Block	Haplotype[a]	Frequency	T (%)	Individual p-value	Permutation[b] p-value	Block p-value
Block 1 (SNPs 1-5)	GACAA	0.304	49.41	0.914	1	
	GGCAG	0.248	62.61	***0.022***	0.157	
	GACGG	0.163	39.68	0.102	0.565	0.055
	AACAG	0.153	54.39	0.508	0.998	
	GGTAG	0.099	31.25	***0.034***	0.271	
	GACAG	0.02	62.65	0.456	0.994	
Block 2 (SNPs 6-7)	CC	0.476	48.69	0.781	1	
	AT	0.287	37.05	***0.015***	0.12	***0.001***
	CT	0.23	69.18	***0.0007***	***0.005***	
Block 3 (SNPs 8-9)	AT	0.441	54.53	0.351	0.979	
	AC	0.286	46.7	0.543	0.999	0.638
	GT	0.274	47.73	0.67	1	

T (%): Transmitted/(transmitted + untransmitted). Significant values ($p < 0.05$) are indicated in bold italic
[a] All possible combinations of haplotypes with frequency > 0.01.
[b] 10,000 permutations.

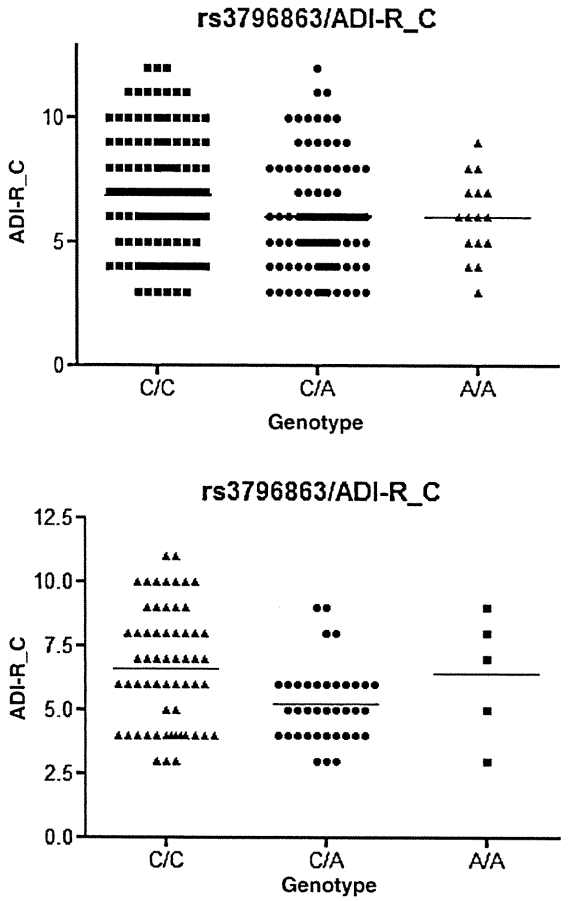

Fig. 3. Comparison of the distribution of ADI-R_C scores of autistic individuals across the C/C, C/A, and A/A genotypes of rs3796863 in 252 trios (A) and HFA trios (B). Significant variation was observed in the distribution of ADI-R_C scores between the three groups, in the 252 trios and HFA trios. The variation in the distribution of ADI-R_C between the C/C and C/A groups was significant at the 0.05- and 0.01-levels in the 252 and HFA trios, following *post hoc* pairwise comparison with Bonferroni's method. One-way analysis of variance (ANOVA), followed by *post hoc* pairwise comparison with Bonferroni's test, was used to examine the variability in the distribution of ADI-R phenotypic data (ADI-R_A, ADI-R_BV, ADI-R_C, ADI-R_D) across the homozygous and heterozygous genotypes of SNPs that showed significant associations in single SNP TDT.

The 2nd haploblock including SNPs06 and 07, showed a strong association ($p = 0.001$) in the HFA subgroup (Fig. 4 and Table 5). In this block, the haplotypes AT ($p = 0.015$) and CT ($p = 0.0007$) showed significant associations; the association shown by CT remained significant (permutation $p = 0.005$), while that of AT was not significant (permutation $p = 0.12$), by permutation. There was an overtransmission (69.18%) of the CT haplotype, with the C allele of SNP06. The C allele of SNP06 also showed overtransmission (54.52%) in single SNP TDT.

The haplotypes of the 3rd haploblock, which included SNPs 08 and 09, did not show any association with HFA. None of the haplotypes in the 252 trios showed significant association.

3.2. Mutation analysis

Next, we performed mutation and/or exonic SNP analysis in *CD38* (Fig. 2A) in cohort 1. The C3139T polymorphism in exon 1 led to an arginine-to-cysteine substitution at codon 47, the C4693T (rs1800561) polymorphism in exon 3 that led to an arginine-to-tryptophan substitution at codon 140 (R140W), and the C6900T polymorphism in exon 7 led to a serine-to-leucine substitution at codon 264. Two others mutations [C4092T (SNP14) and A5346C (rs1800051)] were synonymous.

A weak association was detected for rs1800561 (R140W) in adult ASD patients (average age = 22.8 ± 7.6 years old; allele frequency = 0.052) compared with controls (average age 34.1 ± 4.3 years old; allele frequency = 0.006) ($p < 0.05$) in cohort 1. Therefore, we focused only on the rs1800561 (R140W) polymorphism in case–control cohort 2 (Table 1) from 3 Japanese sites and genotyped in the same platform. Of 301 Japanese ASD subjects with the average age of 11.9 ± 6.7 years old, 13 male (but no female) patients were heterozygous for R140W (allelic frequency, 0.022). In 417 unscreened control subjects without ASD, 10 males and 7 females were heterozygous for the mutation and one female was homozygous (allelic frequency, 0.023). We failed to replicate the association in the larger sample set (cohort 2) ($\chi^2 = 1.20$, $p < 0.3$). Furthermore, although we detected the SNP in 5 of the 150 Korean controls, it was not detected it among the 16 Korean patients (cohort 3 in Table 1) or the 263 Caucasian patients (cohort 4 in Table 1).

3.3. rs1800561 (R140W) SNP in families

In the course of our studies of the rs1800561 SNP (R140W), we identified three families (cohort 1) in which ASD appeared to relatively segregate as a dominant trait (Fig. 5). In these families, fathers, brothers, and other relatives of 3 probands (two autistic (2-II-1 and 3-III-2) and one Asperger (1-III-1)) showed clinically identified ASD or exhibited ASD traits. AQ scores for the brothers of two probands (1-III-2 and 3-III-1) fulfilled the criteria (score >28) for ASD (Asperger disorder).

Twenty-eight family members were available (Fig. 5, not all are shown), and the R140W heterozygous SNP was found in 18 subjects whose ages ranged from 22 to 86 years old. Out of them, 8 carriers were clinically diagnosed as ASD or with ASD traits (44%). In these pedigrees, no ASD subjects were found without this mutant SNP.

Next, we examined whether mRNA from the mutant allele is expressed in the patients. We prepared blood RNA samples from one subject with the C/C genotype and 3 subjects with C/T genotype. cDNA with 4693C has the *Msp*A1I restriction site. The RT-PCR products from homozygous 4693C/C and heterozygous C/T subjects were digested by *Msp*A1I. RT-PCR products from the C/C subject gave two (digested) bands, while those from the C/T subjects gave 3 bands with an additional undigested one (data not shown). Furthermore, sequencing of RT-PCR products of the C/T samples confirmed the existence of the SNP. These results show that the mutant (W140) allele was transcribed and expressed in the 3 probands.

3.4. Plasma OT and vasopressin levels

Plasma OT levels of ASD probands with the W140 allele (79.3 ± 14.9 pg/ml; $n = 3$) were significantly lower than those of ASD subjects without the W140 allele in cohort 1 (147.7 ± 15.0 pg/ml; $p < 0.01$, $n = 26$; Fig. 6). The OT levels in ASD patients with the W140 allele were significantly lower than those in control subjects with the R140 allele (198.2 ± 24.7 pg/ml; $n = 100$; $p < 0.01$). Only one control subject with the W140 allele was available for plasma OT measurement, and the value was 174.7 pg/ml. The OT levels in ASD patients with the R140 allele were almost equivalent to those in control subjects with the R140 allele, suggesting that allele (C/T) status may be a unique determinant for the plasma OT level among multiple confounding factors. In contrast, plasma AVP levels were slightly higher in the probands with the W140 allele than in ASD patients with the R140 allele (38.9 ± 3.8 pg/ml, $n = 3$, *vs.* 26.9 ± 5.0 pg/ml, $n = 26$; $p < 0.05$) (Fig. 6).

Finally, one proband (3-III-2) aged 23 years, diagnosed with autism at the age of 3 years and 9 months, began nasal OT adminis-

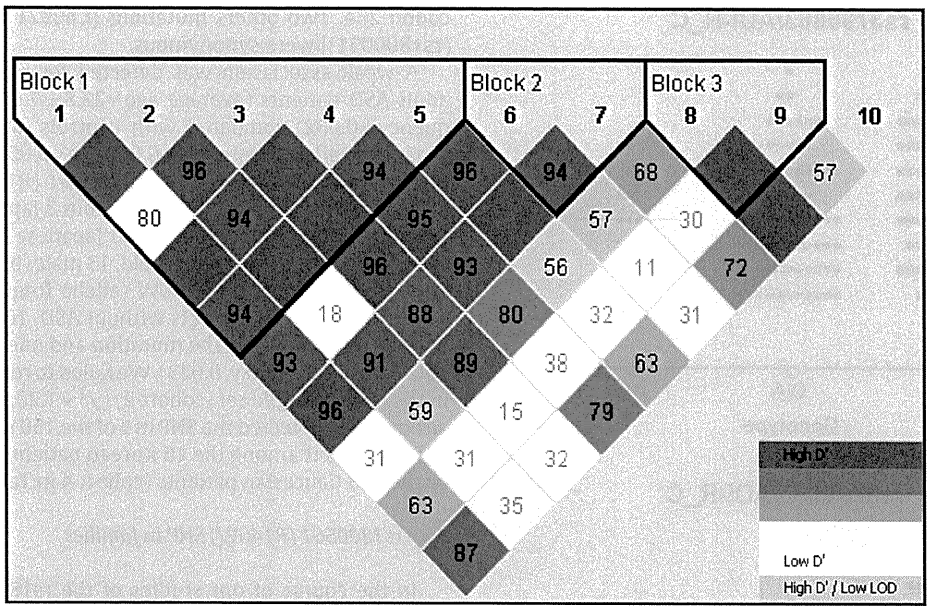

Fig. 4. LD structure of *CD38* based on *D'* values calculated from HFA trios. A linkage disequilibrium (LD) plot was constructed using the *D'* (linkage disequilibrium coefficient; Ranade, 2001) pairwise LD values between markers, estimated using the Haploview software. Based on the LD structure of the gene, haplotype associations were examined; all the haplotypes with frequency >0.01 were included for the association test.

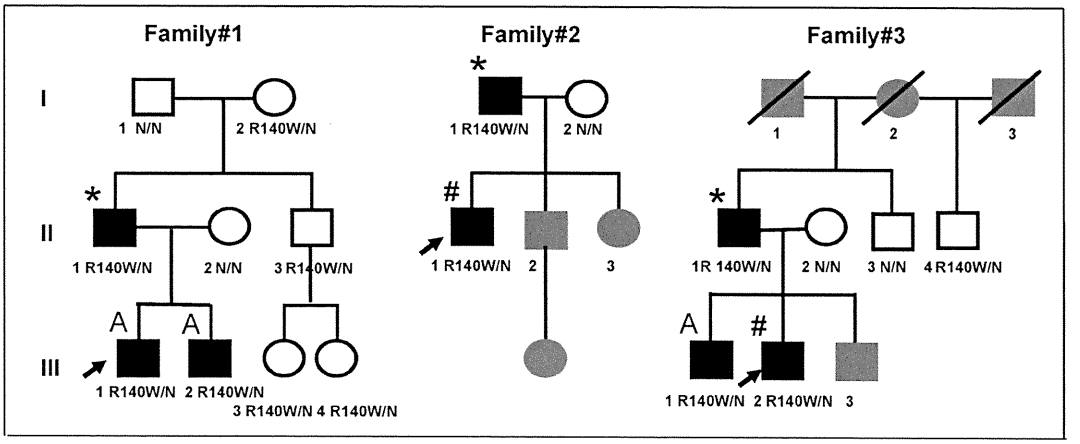

Fig. 5. Pedigrees of the 3 ASD probands carrying the W140 allele. Squares and circles represent male and female family members, respectively. Black squares represent those with ASD or ASD trait. A slash mark through symbols indicates the subject is deceased. The allele status is indicated under the symbols: N/N, two normal alleles; R140W/N, one mutant and one normal allele. Gray symbols indicate undetermined (no DNA available for analysis). The subjects are identified by Arabic numbers, and the generation by Roman numerals. The arrow indicates the proband. (*) Autism trait; (#) Autism; (A) Asperger disorder.

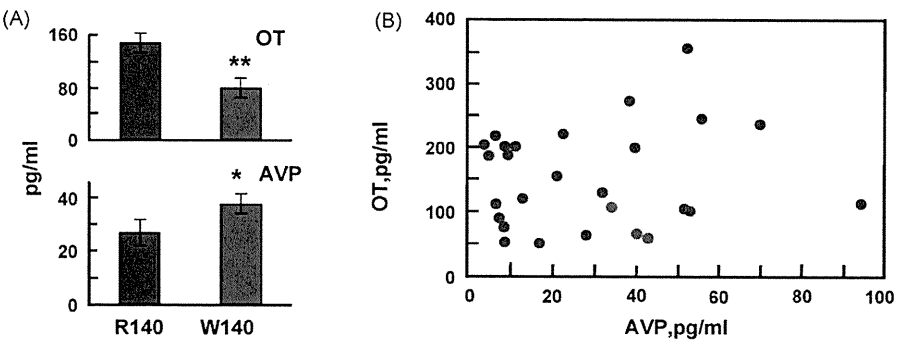

Fig. 6. Plasma oxytocin and vasopressin levels in ASD subjects. Bar graph (A) and Scatchard plot (B) of plasma concentrations of OT and AVP levels in 29 ASD patients in cohort 1 with (red) or without (green) the W140 allele. Mean ± S.E.M. *$p < 0.05$; **$p < 0.01$ (one-way ANOVA).

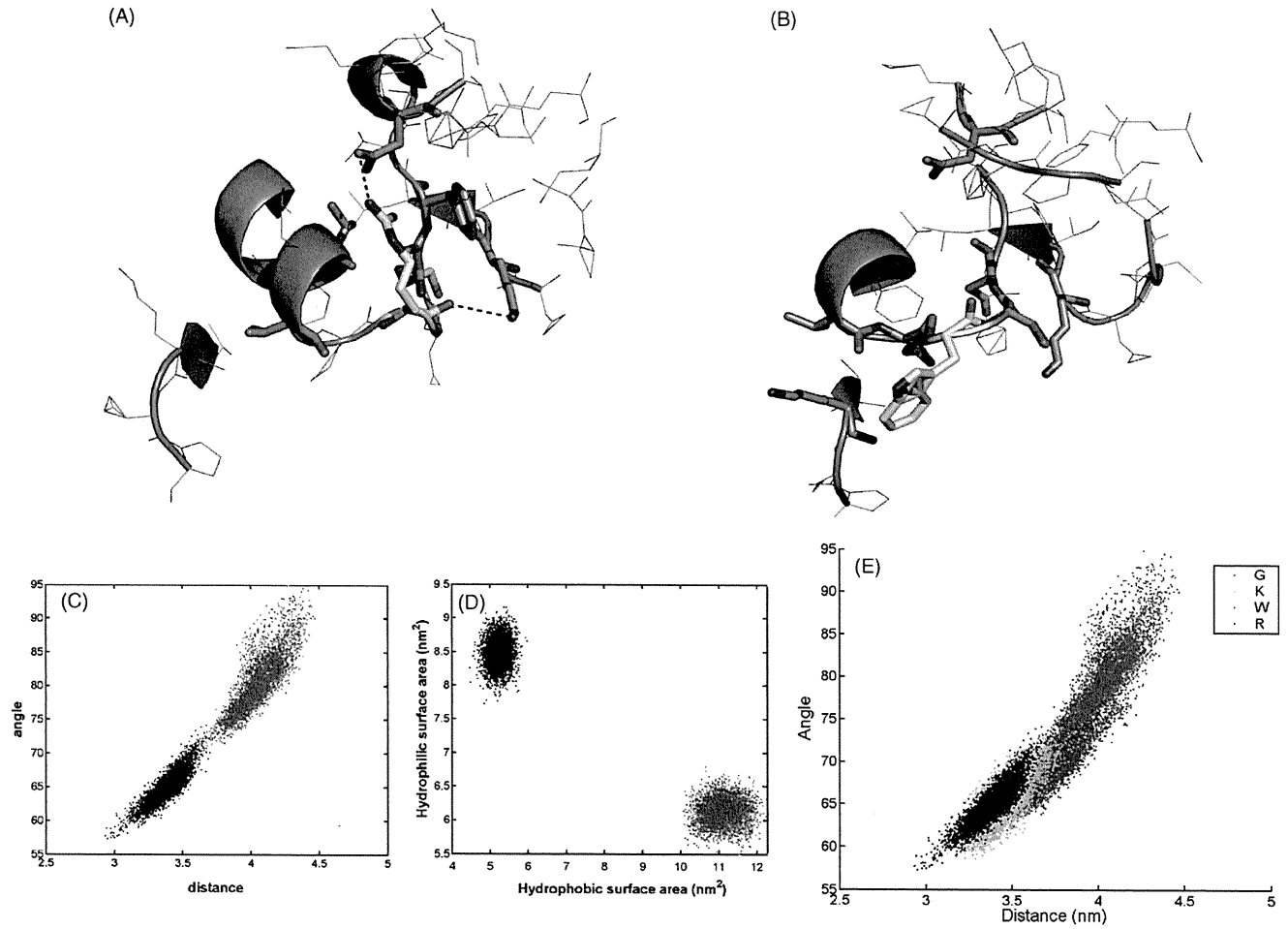

Fig. 7. Molecular structure of CD38. The interactions between R140 (A) or W140 (B) and nearby residues in CD38 protein are shown. Hydrogen bonds are shown as dashed lines. The protein residues are colored as follows: C, blue except the 140 residue (yellow); N, blue; O, red. R140 and W140 are packed into the helix, and the packing conformation seems stable. The W140 protein has an outward conformation and fewer interacting residues than the wild-type protein. (C) Distribution of distance and angle between domains. The distance between Q171 and S213 and the angle between Q171, G113 (hinge), and S213 are plotted based on domain analysis. The R140 structures are shown as black dots and the W140 as red dots. Each point representing a conformation is from MD simulation (5–9 ns). The mutant structure has a more the open conformation than the wild-type and a slightly larger degree of variation. (D) Solvent-accessible surface areas. The active site properties are significantly different between the mutant and wild-type. (E) Distribution of distance and angle between domains during 5–9 ns MD simulation as in (C): R140, black; W140, red; K140, green; and G140, blue. K140 shows almost the same distribution as the wild-type. G140 shows a slightly different distribution, but less than W140.

tration twice a day at home with parental assistance in June 2008. The immediate effect of OT was obvious after the first trial, in which he was no longer boisterous after awaking early in the morning. This quieting on awaking has been maintained for more than 12 months. He showed improvements in eye contact behavior with smiling and answering to yes/no questions in his daily life. There were no significant adverse effects.

4. Discussion

Here, we demonstrated that one rs3796863 (C > A) SNP of *CD38* showed significant association with U.S. but not Japanese high-fiunctioning autism (HFA) patients, with a cutoff at IQ > 70. Based on the results of SNP- and haplotype-TDT analyses, the A allele of rs3796863 of *CD38* may be considered a protective allele and the C allele as a risk allele for U.S. HFA cases. As the allele frequency is about 0.3, this variant is common. Very recently, common variants on 5p14.1 between neural cadherin 10 and 9 have been reported to associate with ASD (Wang et al., 2009). As rs3796863 is an intronic SNP, the functional importance of this SNP remains to be determined. There were significant variations in the distribution of ADI-R_C scores (restricted, repetitive, and stereotyped patterns of behavior) between the C/C, C/A, and A/A genotypes of this SNP in 252 trios and HFA trios. The second haploblock of *CD38*, which included rs3796863, showed a significant association with HFA. The association shown by the CT haplotype of second haploblock remained significant by permutation analysis. This common variant (Arking et al., 2008) may contribute to the genetic susceptibility of HFA, in addition to other susceptiblity genes for HFA (Weiss and Arking, 2009; Wermter et al., 2010).

SNP (rs1800561) of *CD38* was reported in Japanese (allele frequency, 0.035) and Han Chinese (0.01) but not in European or African control populations in the online SNP database. However, recently, it was detected in Polish Caucasians, where 3 healthy controls out of 500 yielded an allele frequency of 0.003, and 21 W140 carriers were found among 439 B-cell chronic leukocytic leukemia patients (frequency, 0.024) (Jamroziak et al., 2009). An Italian study indicated one carrier among 25 healthy controls (frequency, 0.02; cohort 4) (Mallone et al., 2001). We found 68 carriers of the T genotype among 1384 Japanese, and they included controls, ASD patients, and family members of ASD patients. We also detected this genotype in 5 of 150 Koreans non-ASD controls with diabetes (frequency, 0.017; cohort 3) (Table 1), indicating that the polymorphism is more common among Asians than Caucasians.

The biological relevance of the inheritance patterns of the R140W allele was unclear in the context of ASD. The male offspring of W140 carriers seem to have a higher risk of ASD than females. Why the effect of the W140 variant on development of ASD varies with sex is unknown, but females may have more protective factors and/or weaker risk factors related to female hormones or OT. Plasma OT levels were lower in W140 allele ASD carriers than R140 ASD carriers. However, taking into account the results that the W140 allele is not associated with ASD in general population, we can only say that the W140 allele is deemed to have a role in decreased plasma OT levels, but not in AVP levels, regardless of disease status. This scenario is expected from our prior observation in *Cd38* knockout mice (Jin et al., 2007), in which the plasma OT but not AVP level was differentially decreased.

Our finding that one proband (3-III-2 in Fig. 5) with the W140 allele had been receiving intranasal OT was unexpected. His social behavior showed some improvement after the first administration of OT, and this improvement has been maintained for more than 12 months. The observed effects are in accordance with those reported previously for OT: improved mind- or emotion-reading and social memory, increased eye contact, and positive communication (Hollander et al., 2007; Domes et al., 2007; Guastella et al., 2008a,b, 2010). However, to our knowledge, this is the first report of a long-term therapeutic effect of OT on the social deficits in ASD, suggesting that it may benefit a broader group of patients, disorders, or typical adults and individuals carrying this rare allele.

We conducted a preliminary structural analysis of CD38 and various mutant proteins by Dyndom and MD simulation to analyze domain motion (Hayward and Lee, 2002) (Fig. 7). W140-CD38 had a completely different conformation than R140-CD38 (Fig. 7A and B). The charge change at the packed site caused by the R→W substitution was likely the primary reason for the outward conformation of the mutant protein. The mutant structure is more open and has a slightly larger degree of variation (Fig. 7C and E). Fig. 7D shows the solvent-accessible surface area of the active site of the mutant protein altered by the closure motion, significantly changing its properties. Thus, W140 CD38 showed changes that may affect the substrate binding affinity and eventually enzyme activity. The amino acid substitution can cause severe perturbations of the predicted protein structure in comparison with wild-type human (R140), rabbit (K140), and mouse (G140) CD38. In CHO cells, in fact, the W140-CD38 protein possesses only one third of the ADP-ribosyl cyclase activity of R140-CD38 (Yagui et al., 1998). Moreover, social amnesia was not rescued by local re-expression of human W140-CD38 in the hypothalamus in the *Cd38* null mice (Jin et al., 2007). Taken together, these observations indicate functional abnormality of W140-CD38.

In conclusion, despite their statistical limitations, our results suggest that the rs3796863 SNP may contribute to genetic susceptibility to HFA in U.S. but not necessarily in Japanese subjects (at least to the limits of our current analysis). Our results call for functional and expression assay to assess the biological effects of the variant. Furthermore, the W140 allele could be a potential risk factor for a subset of Japanese ASD patients, *i.e.*, males with low blood OT levels. Patients in this subgroup are candidates for a clinical trial of OT treatment, although further systematic case–control investigations are required to verify its effects.

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ORIGINAL ARTICLE

Association of the oxytocin receptor (*OXTR*) gene polymorphisms with autism spectrum disorder (ASD) in the Japanese population

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The oxytocin receptor (*OXTR*) gene, which is located on chromosome 3p25.3, has been implicated as a candidate gene for susceptibility of autism spectrum disorder (ASD). Positive associations between *OXTR* and ASD have been reported in earlier studies. However, the results were inconsistent and demand further studies. In this study, we investigated the associations between *OXTR* and ASD in a Japanese population by analyzing 11 single-nucleotide polymorphisms (SNPs) using both family-based association test (FBAT) and population-based case–control test. No significant signal was detected in the FBAT test. However, significant differences were observed in allelic frequencies of four SNPs, including rs2254298 between patients and controls. The risk allele of rs2254298 was 'A', which was consistent with the previous study in Chinese, and not with the observations in Caucasian. The difference in the risk allele of this SNP in previous studies might be attributable to an ethnic difference in the linkage disequilibrium structure between the Asians and Caucasians. In addition, haplotype analysis exhibits a significant association between a five-SNP haplotype and ASD, including rs22542898. In conclusion, our study might support that OXTR has a significant role in conferring the risk of ASD in the Japanese population.
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Keywords: ASD; case–control; family-based association; *OXTR* gene; SNP

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopment disorder characterized by impairment in social interaction and communication, as well as restricted and stereotyped behaviors and interests.[1,2] In its broad definition, it affects 1 in every 152 children[3,4] and the ratio between affected male and female is 4:1. Owing to its early onset (with usual appearance of the symptoms before the age of 3 years) and lack of effective therapeutic strategy, ASD causes huge emotional and financial burden to the patients, their families and the society.

Twin studies show that the concordance rate of ASD in monozygotic twins is 70–90%, but only 0–10% in dizygotic twins.[5] Family studies indicate that the prevalence of ASD in siblings of the affected individuals is 50–100 times higher than the general population.[6] Lines of compelling evidence have firmly established that ASD has a strong genetic component; however, the underlying mechanism remains elusive, which might be due to the genetic heterogeneity and complexity.

In the past decades genome-wide linkage studies and genetic association studies have been performed to identify susceptibility genes for ASD, in which a number of potential candidate genes have been implicated.[7–10] However, all known genetic variations can only account for 10–20% of the ASD patients.[11]

Several hypotheses have been proposed to explain the causes of ASD.[12–14] In these hypotheses, the oxytocin and vasopressin systems are receiving increasing attention because of their special roles in regulating a range of social behaviors, including social recognition, pair bonding and maternal care, which were observed in animal studies.[2,15] Mice lacking the oxytocin gene fail to develop social memory.[14] *OXTR* knockout mice showed several aggravations in social behaviors including mother–offspring interaction.[16] In human, autistic children tended to have a lower plasma oxytocin level compared with healthy individuals.[17] Oxytocin infusion might reduce repetitive behaviors in autistic patients.[18] In addition, the oxytocin receptor (*OXTR*) gene is located at 3p25.3, in the chromosomal region, which has been suggested for autism in a genome-wide linkage study.[19] On the basis of these findings, it is reasonable to postulate that the dysfunction of oxytocin system may be associated

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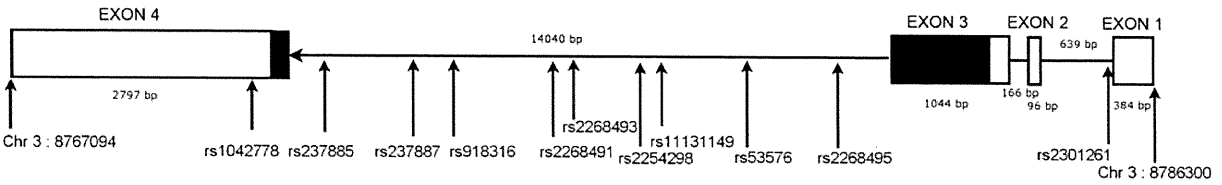

Figure 1 Schematic diagram of the 11 SNPs of the OXTR genotyped in the current study. Each block indicates an exon. White and black blocks show untranslated region and coding regions, respectively.

with susceptibility to autism. To date, Chinese and Caucasian studies observed significant genetic associations between *OXTR* and ASD.[20–22] Among them, Wu *et al.*[20] were the first to study the genetic association of single-nucleotide polymorphisms (SNPs) of *OXTR* in autism. They investigated four SNPs in Han-Chinese ASD trios and found that 'A' alleles of rs53576 and rs2254298 were significantly overtransmitted. Two Caucasian studies[21,22] also found the association between *OXTR* and ASD, but the detail of the results was inconsistent compared with the Chinese result. Jacob *et al.*[21] investigated the two SNPs (rs53576 and rs2254298), which were suggested in the Chinese study, and found that the 'A' allele of rs2254298 was undertransmitted, not overtransmitted. No association of rs53576 was observed. Lerer *et al.*[22] found an association of a haplotype comprising rs22254298 and four other SNPs (rs237897–rs13316193–rs237889–rs2254298–rs2268494) with ASD, but as a single SNP, rs2254298 was not associated with ASD (Lerer *et al.*[22]). Yrigollen *et al.*[23] found that rs2268490, which was in the same haploblock of rs2254298, was associated with ASD.

These results require further studies to clarify the role of the *OXTR* polymorphisms in ASD. In this study, we conducted family-based association test (FBAT) and population-based case–control test in Japanese subjects with ASD.

MATERIALS AND METHODS

Subjects

Two sample sets were included in this study. A set consists of 217 families including 223 affected individuals (189 males and 34 females, age=18.35 ± 9.5 years (mean ± s.d.)) with their parents. Among the 223, 207 were diagnosed with autistic disorder, 3 with Asperger's disorder and 13 with pervasive developmental disorder not otherwise specified. Another set comprised 65 unrelated autistic disorder cases without parents' sample (57 males and 8 females, age=25.20 ± 4.8 years (mean ± s.d.)). The 282 ASD samples for case–control association study are the combination of 217 independent cases from the family samples and 65 unrelated samples. The diagnoses were made by two or more senior child psychiatrists through interviews and reviews of clinical records, according to the DSM-IV criteria (American Psychiatric Association, 1994). Controls consisted of 440 unrelated healthy Japanese subjects (272 males and 168 females, age=40.9 ± 9.7 years (mean ± s.d.). The cases were recruited from the outpatient clinics of the Departments of Psychiatry, Tokyo University Hospital and Tokai University Hospital, and seven day care facilities for subjects with developmental disorders. The hospitals and facilities were located around Tokyo, Nagoya and Mie. The controls were recruited around Tokyo, without any psychiatric disorder disturbing their work function. The Mini-International Neuropsychiatric Interview[24] and other questions were administered in the recruitment of controls to exclude those who had present or lifetime history of mental disorders. All the patients and controls were ethnically Japanese, with no parents or grandparents of ethnicity other than Japanese. Objective of the study was clearly explained, and written informed consent was obtained from all control subjects or parents of the affected individuals. The consent was also obtained from the affected individuals when they were able to follow the explanation. The study was approved by the Ethical Committee of the Faculty of Medicine, the University of Tokyo.

Genotyping and selection of the SNPs

Genomic DNA was extracted from the peripheral blood using the standard phenol–chloroform method for the family samples and unrelated case samples. For the control samples, genomic DNA was isolated from whole blood by using Wizard Genomic DNA Purification Kit.[25]

On the basis of the genotype data in the Japanese population from the HapMap Project, we selected pair-wise tag-SNPs with minor allele frequencies >0.05 and a threshold of r^2=0.8 in the *OXTR* region including promoter and other areas, using the Tagger program implemented in Haploview. Intron 3 was the most densely mapped as shown in Figure 1. In addition, we studied three other SNPs, rs2268493, rs53576 and rs1042778. These were reported to be associated with ASD in the previous studies.[20–23] Thus, a total of 12 SNPs were selected. All SNPs were genotyped by using TaqMan genotyping platform. The TaqMan probes were ordered from the Assays on Demand system of the Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Genotyping was performed in 5-μl system containing 2.5 μl of TaqMan Universal PCR Master mix, 0.25 μl of 20× TaqMan probe and 1 μl genomic DNA using Roche LightCycler 480 II (Roche Diagnostics, Tokyo, Japan). Allele calling was performed using LightCycler CW 1.5 software (Roche Diagnostics).

Statistical analysis

Hardy–Weinberg equilibrium was tested in control subjects and parent subjects in family samples by χ^2-test using Haploview software ver 4.1.[26,27] FBATs were conducted using FBAT ver 1.7.2.[28,29] Case–control association analyses were performed by standard χ^2 test using the PLINK program.[30] Odds ratio for each allele and the 95% confidence intervals were also calculated. To correct the multiple testing, Bonferroni correction was implemented based on the number of SNPs analyzed in the *OXTR*. We set the critical *P*-value for a positive association at 0.0045.

Pairwise linkage disequilibrium (LD) was derived from genotyping data of control samples using the confidence intervals method (the Gabriel method) provided by HaploView. The same software was used to estimate haplotype frequency and haplotype *P*-value. The empirical *P*-values were generated on the basis of 10 000 permutation tests. The global *P*-value for each haploblock was calculated by the UNPHASED software (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased).

RESULTS

Genotype distribution of one SNP (rs2268490) was significantly deviated from Hardy–Weinberg equilibrium in control subjects (*P*-value <0.001), and this SNP was excluded from the analysis. No significant deviation from Hardy–Weinberg equilibrium was observed in parents of the family samples for any SNPs. We performed the Mendelian inheritance check for the family samples. Two families showed inconsistency and were also excluded.

Single-marker association analysis

The FBAT did not reach the statistical significance (*P*-value <0.05) in this study (Table 1).The results of SNP analyses based on case–control test are summarized in Table 2. Statistically significant association with ASD was observed in four SNPs, including rs237887, rs2268491, rs2254298 and rs2268495 (*P*=0.023, 0.004, 0.001 and 0.032, respectively). *P*-value for rs53576 was margined 5% (*P*=0.053). Two SNPs

(rs2268491, rs2254298) remained significant after Bonferroni correction for the multiple testing.

LD structure

The standardized measure of LD denoted as r^2 was calculated for all pairs of SNPs .Two haploblocks were suggested according to confidence intervals method (Figure 2). LD block 1 consisted of two SNPs (rs237885, rs237887), which correspond to exon 4 untranslated region, and exon4–intron 3 boundary region. Block 2 that spans about 4.7 kb comprises five SNPs located in intron 3. Of these five, two SNPs (rs2268491 and rs2254298), which were significantly associated with ASD in this study, are in perfect LD ($r^2 > 0.95$). Two unassociated SNPs (rs2268493 and rs11131149) were in low LD with the two associated SNPs, but they were in perfect LD with each other.

We then carried out haplotype analysis within these two haploblocks and conducted permutation test. For haplotype analysis positive association was noted in both haploblocks (Table 3). Two out of three haplotypes in the block 1 accounted for 85% in controls and showed nominal association with ASD with P=0.029 and 0.042, respectively. One of the five-SNP haplotypes in the block 2, which carries 'A' allele of rs2254298, showed a significant association with ASD (P=0.004). The global P-value for the two haploblocks is 0.0093 and 0.00187, respectively. By permutation test, a significant association with ASD was observed for the five-SNP haplotype in the block 2, but not for the haplotypes in the block 1.

DISCUSSION

The case–control analysis of this study generated significant associations between SNPs in *OXTR* and ASD in the Japanese population, whereas family-based analysis did not provide a support for the association. In the case–controls analysis, 4 SNPs out of 11 were significantly associated with ASD at 5% or stronger level of significance. The most significantly associated SNP was rs2254298 with 'A' as the risk allele. Haplotype analysis also provided a support for the association of the five-SNP haplotype in intron 3, containing rs2254298, with ASD. These results may suggest the potential role of *OXTR* in ASD, as implicated in the previous studies.[20–22]

In contrast to the case–control analysis, we did not obtain a support for the association in the family-based analysis. This could be due to the insufficient power with limited numbers of informative families. Regarding the two interesting SNPs (rs2254298 and rs53576), 'A' alleles were preferentially transmitted to the affected individuals (Z=0.862 in 124 informative families for rs2254298 and Z=0.931 in 128 informative families for rs53576). This could be consistent with the

Table 2 Allele frequencies of 11 OXTR SNPs in 280 unrelated ASD cases and 440 controls

	SNPs (minor/major alleles)	*MAF (case)*	*MAF (controls)*	P-*value*	*OR (95% CI)*
1	rs1042778 (T/G)	0.098	0.097	0.931	1.016 (0.709–1.456)
2	rs237885 (G/T)	0.247	0.292	0.068	0.799 (0.627–1.017)
3	rs237887 (A/G)	0.378	0.439	0.023	0.776 (0.624–0.966)
4	rs918316 (C/T)	0.204	0.222	0.424	0.899 (0.693–1.167)
5	rs2268491 (T/C)	0.325	0.255	0.004	1.405 (1.112–1.775)
6	rs2268493 (C/T)	0.152	0.160	0.694	0.942 (0.701–1.266)
7	rs2254298 (A/G)	0.335	0.256	0.001	1.459 (1.156–1.841)
8	rs11131149 (A/G)	0.165	0.172	0.743	0.953 (0.714–1.271)
9	rs53576 (G/A)	0.333	0.383	0.053	0.802 (0.642–1.003)
10	rs2268495 (A/G)	0.186	0.235	0.032	0.747 (0.572–0.976)
11	rs2301261 (A/G)	0.115	0.085	0.065	1.395 (0.979–1.988)

Abbreviations: ASD, autism spectrum disorder; CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; OXTR, oxytocin receptor.

Table 1 Results of the family-based association test (FBAT) of 11 SNPs in 215 ASD families

No.	*SNPs*	*Position*	*Allele*	*Number of families*	S	E(S)	Z	P-*value*
1	rs1042778	Chr3: 8769545	G	57	82	85	−0.739	0.46
			T	57	36	33	0.739	0.46
2	rs237885	Chr3: 8770543	G	115	81	82	−0.167	0.868
			T	115	157	156	0.167	0.868
3	rs237887	Chr3: 8772042	A	133	115	123.5	−1.278	0.201
			G	133	161	152.5	1.278	0.201
4	rs918316	Chr3: 8773181	C	93	63	69	−1.105	0.269
			T	93	127	121	1.105	0.269
5	rs2268491	Chr3: 8775398	C	119	150	154	−0.636	0.524
			T	119	94	90	0.636	0.524
6	rs2268493	Chr3: 8775840	C	82	50	49.5	0.102	0.919
			T	82	120	120.5	−0.102	0.919
7	rs2254298	Chr3:8777228	A	124	100	94.5	0.862	0.389
			G	124	156	161.5	−0.862	0.389
8	rs11131149	Chr3: 8777852	G	88	128	127.5	0.098	0.922
			A	88	54	54.5	−0.098	0.922
9	rs53576	Chr3: 8779371	A	128	156	150	0.931	0.352
			G	128	104	110	−0.931	0.352
10	rs2268495	Chr3: 8782535	A	97	62	70	−1.403	0.161
			G	97	142	134	1.403	0.161
11	rs2301261	Chr3: 8785896	G	62	89	91.5	−0.602	0.547
			A	62	37	34.5	0.602	0.547

Abbreviations: ASD, autism spectrum disorder; *E(S)*, expected value of *S* under the null hypothesis (that is, no linkage or association); *S*, test statistics for the observed number of transmitted alleles; SNP, single-nucleotide polymorphism; *Z*, FBAT *Z* score of multiallelic test for the allele effects.
Number of families: number of informative families (that is, families with at least one heterozygous parent).

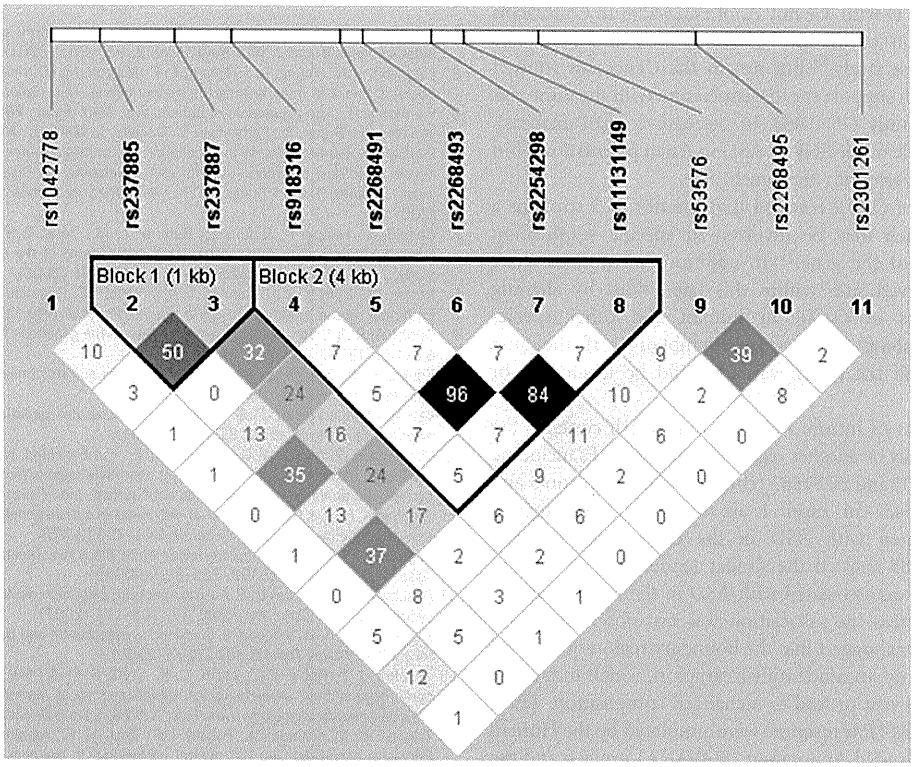

Figure 2 Pairwise LD analysis. LD between SNPs in *OXTR* was derived from genotyping data of control samples. The white bar above the SNP names represents the location of each SNP along the gene. The number within each square indicate the r^2-values.

Table 3 Haplotype analysis of SNPs in two LD blocks by permutation

(Block 1)		*Estimated frequency*			
rs237885	*rs237887*	*Cases*	*Controls*	P-*value*	*Permutation* P-*value*
G	A	0.238	0.290	0.029	0.162
T	G	0.613	0.559	0.042	0.252
T	A	0.140	0.150	0.581	0.999

(Block 2)					*Estimated frequency*			
rs918316	*rs2268491*	*rs2268493*	*rs2254298*	*rs11131149*	*Cases*	*Controls*	P-*value*	*Permutation* P-*value*
T	T	T	A	G	0.314	0.245	0.004	0.018
C	C	T	G	G	0.188	0.212	0.252	0.929
T	C	C	G	A	0.149	0.155	0.739	1.000
T	C	T	G	G	0.303	0.356	0.040	0.241
T	C	T	G	A	0.020	0.016	0.601	1.000

Abbreviations: LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.

Chinese finding,[20] although the results did not reach the level of statistical significance.

Regarding the association of the intron 3 region of *OXTR*, previous studies observed controversial results.[20–22] In a Chinese study, the 'A' allele of rs2254298 was risk for ASD,[20] whereas the 'A' allele was protective for ASD in the Caucasians.[21] In addition, a Jewish study[22] found a haplotype, which contained rs2254298 with the 'A' allele, as protective. This study observed the association of the 'A' allele with ASD as a risk allele with the *P*-value of 0.001 in the case–control analysis, which is consistent with the Chinese investigation. This suggests that the 'A' allele of rs2254298 may increase the risk of ASD in the Asians, but not in Caucasians. The SNP rs2254298 itself therefore may not be a real causal variant. Another unknown variant in strong LD with this SNP might be responsible for ASD, in which the

risk allele might be in LD with 'G', not 'A', of rs2254298 in Caucasians and Jewish. Association of another SNP, rs53576, and ASD was observed in the Chinese study,[20] but not in the Caucasian study.[21] This study found a weak support for the association with the same risk allele in the Chinese study (P=0.053, in the case–control analysis). This might suggest a role of the SNP in ASD in Asian populations, but further studies are requested for the conclusion.

A functional study of *OXTR* revealed that the intron 3 contains a genomic element, which may be involved in specific suppression and down regulation of the gene.[31] In addition, we noticed there are two highly conserved sites within this haploblock by aligning homologous sequences of *OXTR* in human and other species using ENSEMBL database (http://www.ensembl.org). If the sites are functionally critical, the causal variant could be located in or close to the sites.

The boundary region of intron 3 and exon 4, which contains the block 1 in this study, may be another region of interest. SNPs including rs237885 (chr3:8770543) and rs237887 (chr3:8769545) in intron 3 and rs1042778 (chr3:8769545) in exon 4 are located in this region. Rs237887 was associated with ASD in the case–control analysis (P=0.023) and rs237885 showed the similar tendency (P=0.068) in this study. Rs1042778 was associated with ASD in the Jewish population (P=0.014),[22] whereas the association was not observed in this study. These observations suggest that the boundary region of intron 3 and exon 4 could contain an unidentified variation, which may cause an alternative splicing event or lead to a changed composition. Three different forms of the *OXTR* transcripts were annotated by the Human And Vertebrate Analysis aNd Annotation (HAVANA) project, and the 4th exon was missing in two forms of the transcripts. *OXTR* mRNAs of different length (3.6 and 4.4 kb) have been also reported.[32] Such kind of variations could be related with the observed association between this region and ASD.

There are some major limitations to the current study. First, we focused on the tag SNPs and the SNPs found associated with ASD in previous studies, but we did not study nonsynonymous SNPs annotated in the dbSNP database. It is not clear how the substitution of the amino acid affects the function of OXTR, therefore, future studies should include these SNPs. Statistical power of this study is 0.79 for the case–control study and 0.59 for family-based association study when assuming genotypic relative risk is 1.65 under dominant model and SNP frequency is 0.1 for significant level α=0.05. Thus small effect might not be detected because of the sample size. Caution was needed to interpret this study that the controls are not age or sex matched to the case subjects. The sex imbalance in case and control subjects might be resolved by analysis confining the subjects to males considering the higher prevalence in male than in the female. The association of rs2254298 was significant after the correction of multiple testing in males (P=0.003, not written in the result). Finally, a caution might be noted that subjects were recruited from two areas of Japan, and the number of the case and control from the two areas were not exactly matched.

In summary, the case–control analysis of this study suggested that *OXTR* might have a role in the development of ASD in the Japanese population. The 'A' allele of rs2254298 may be the risk allele in Japanese, in accordance with the previous Chinese finding.[20] Further studies of *OXTR* with larger sample size and denser markers or direct sequencing may be recommended for the search of the causal variants.

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Original article

Fragile X carrier screening and *FMR1* allele distribution in the Japanese population

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Abstract

Fragile X syndrome (FXS), which is the most common form of familial mental retardation, is caused by the expansion of the CGG repeat in the *FMR1* gene on the X chromosome. Previous studies have suggested that as compared to other populations, Japanese have a lower prevalence of FXS. In addition, in the normal population, there are no carriers who have the premutation allele. We analyzed a total of 946 normal Japanese (576 males and 370 females) and attempted to estimate the frequency of the *FMR1* allele. Within this population, we found that 1,155 alleles were in the normal range (less than 40 CGG repeats) and had a modal number of 27 repeats (35.75%). No carriers with premutations (55–200 CGG repeats) were observed in this normal population. We also identified six intermediate-sized alleles (40–54 CGG repeats), with a reported incidence of 1 in 103 males and 1 in 324 females. However, this allele frequency was different from that previously reported for the Japanese population. Since data from previous studies has suggested that FXS might possibly be associated with the genetic mechanism of autism, we also analyzed the length of the CGG repeats in 109 autistic patients. In all cases the CGG repeat numbers were within the normal range (16–36 repeats) and no individuals presented with expanded premutation or intermediate alleles. This finding indicates that the length of the CGG repeat within the *FMR1* is unlikely to be responsible for autism in Japanese.

Keywords: Fragile X syndrome; *FMR1*; CGG repeat; Premutation allele; Autism

1. Introduction

Fragile X syndrome (FXS) has been reported to be the common cause of inherited mental retardation [1]. Clinically, these patients exhibit mental retardation, macroorchidism, large ears and long faces. In most

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cases, the mental retardation is moderate-to-severe, with frequent occurrences of autistic-like behaviors. Approximately 30% of the individuals with FXS are classified as being within the autistic spectrum [2]. While several reports have suggested there is an association between the FXS and autism, as of yet, no strong evidence has been found that confirms a link to autism [3].

FXS is caused by an expansion of the CGG repeat, which is located in the 5′-untranslated region (5′-UTR) of the first exon of the fragile X mental retardation 1 gene (*FMR1*) at the chromosomal locus Xq27.3 [4]. The number of CGG repeats is highly polymorphic, ranging from 6 to 50 triplets in normal individuals. The expansions with more than 200 repeats, are observed in fragile X syndrome and are named the full mutation. Full mutation results in hypermethylation of the CpG island within the *FMR1* promoter region along with transcriptional silence of the gene. When a premutation (55–200 repeats) is maternally transmitted it can expand to a full mutation. It has been reported that the larger repeats carry greater risks of expansion than the smaller repeats [5]. The intermediate allele (between 40 and 54 repeats) have been shown to be slightly unstable upon transmission [5,6]. A full mutation in a proband were expanded from an intermediate allele over a span of two generations [7]. The intermediate alleles have been termed 'gray zone' alleles [8] and the larger the size the greater the increase in the instability. The American College of Medical Genetics has recommended that intermediate alleles be considered as a possible risk factor for repeat expansion [9]. At the present time, the frequency of the intermediate alleles in the Japanese population remains unknown.

To determine the prevalence of FXS, a previous study examined patients with mental retardation for the full mutation and initially estimated the rate to be 1 for every 4000–6000 males, although this appeared to vary from group to group [10]. More recently, in order to determine more accurate estimations, several studies were performed in the general population and results indicated that 1 out of 113–441 females and 1 out of 813–1674 males were carriers with the premutation alleles [11–14].

In another study that screened for the full mutation, it was found that there was a lower prevalence of the mutation in Japanese than in other populations [15]. In previous screenings among the normal Japanese population, no premutation allele were found in two different studies, one that examined 824 X chromosomes [16] and one that examined 826 X chromosomes [17]. These results were lower than that observed in Caucasians. Based on these findings, it appears that the prevalence of FXS and allele distribution in Japanese is different from other populations.

In this study, we focused on the CGG repeat length for use in both detecting the intermediate and premutation alleles among the general population. Furthermore, we also analyzed the length of the CGG repeats and their potential involvement for autism in Japanese.

2. Materials and methods

2.1. Samples

A total of 946 normal Japanese samples (576 males and 370 females) collected by the Pharma SNP consortium (PSC) were analyzed [18–20]. PSC control population represents those who voluntarily took part in the project in response to public internet invitation for collecting healthy control population against major illness such as diabetes, hypertension, dementia, cancer, or allergic diseases. Although the socioeconomical and educational condition cannot be specified, they represent self-declaring Japanese control population in Tokyo area with the absence of major illness confirmed by the physician.

Samples from 109 autistic Japanese patients were collected at Tokai University and The University of Tokyo. All autistic patients were diagnosed using the DSM-IV and ICD-10 criteria by two child psychiatrist. IQ score was evaluated and each 13, 13, 12 and 17 patients were more than 70, 69–50, 49–35 and below 34, respectively. The score were not evaluated in 54 patients. Total DNA was extracted from peripheral blood or lymphoblasts, as has been previously reported [15]. The Ethical Committees of the Faculties of Medicine at Tottori University, Tokushima University, Tokai University and Tokyo University approved the study protocol.

2.2. CGG repeat analysis

Analysis of the CGG repeat of the FMR1 gene was performed using a previously reported method [15] with minor modification. For relatively short size repeats, we amplified the repeat using the Cy5-labeled forward primer, with the amplicons analyzed using an ALFred DNA automated sequencer (Amersham Biosciences). PCR method 2 (hybridization method) was used to detect the expanded repeats [15]. We already confirmed PCR method 2 (hybridization method) enough to detect the expanded allele compared with a Southern blotting for the limited amount of the DNA. By the hybridization method, we could detect the normal to full mutation allele. All PCR products were analyzed using an ALFred DNA automated sequencer and PCR method 2 (hybridization method).

2.3. Statistical analysis

To statistically compare the distribution of the CGG repeat length between our current study and other previously published reports, we analyzed the data on a clumped 2×2 table using the CLUMP software [21].

3. Results

3.1. FMR1 allele frequency in the general population

For the *FMR1* (CGG)n allelic expansion, we analyzed 946 normal Japanese samples (576 males and 370 females). A total of 1161 alleles, (513 male and 324 female samples), were considered appropriate for amplification of the CGG repeat region of the *FMR1* gene. We could not amplify the repeat region from 155 alleles, (63 males and 46 females). Results indicated there were no carriers with an allele for full mutation or premutation. As seen in Table 1 and Fig. 1, all of the detected alleles were within the normal range (≤50 CGGs). The number of CGG repeats ranged from 8 to 50, with a modal number of 27 (35.75%), a second peak at 26 (19.29%), and a minor peak at 34 (5.25%). Jointly, 26–28 repeats were found in 844 alleles (72.61%). Intermediate size was defined as 40–50 repeats, and a total of 6 alleles were found within this range (5 males and 1 female). The overall normal allele frequency was 99.48% (1155/1161) and the intermediate allele prevalence was 0.52% (6/1161), i.e., 1:194 X chromosomes. Allele distribution observed in this study was significantly different from previous analyses that used the CLUMP software to examine Caucasian [4] (730.53, $p = 0.000010$), Mexican [22] (563.14, $p = 0.000010$) and other Japanese [16] (505.23, $p = 0.000010$) populations.

3.2. Analysis in autistic patients

Among the 109 patients (116 alleles), no expanded or intermediate alleles with more than 50 repeats were found (Table 2). All affected children had normal alleles that ranged from 16 to 36 with the first peak at 26 (38.79%). As compared to the general population, there were no significant differences noted among the *FMR1* allele frequencies. We also analyzed the patient's parents (106 mothers and 106 fathers, total 318 alleles), and found no premutation allele carriers (data not shown).

4. Discussion

In this study, we used the DNA samples from collected by the Pharma SNP consortium (PSC), a DNA Bank. The samples were kept relatively longer than the usual DNA testing. The amplification of the CGG repeat region is sometimes difficult if the sample is not fresh. Only the limited DNA samples were available and we could not amplify the repeat region from 155 alleles.

In previously reported studies, the CGG repeat allele frequencies differed ethnically [16,22] from the data reported by Arinami et al. for the normal Japanese population. The data reported by Arinami et al. also differed from our current results. The modal repeat numbers reported by Arinami et al. were 28 (40.5%), 29 (30.8%) and 35 (7.8%) while our numbers were 27 (35.8%), 26 (19.3) and 28 (17.6). The reason for this difference might

Table 1
Distribution of *FMR1* allele in normal Japanese population.

CGG	Female	Male	%	CGG	Female	Male	%
<Normal range>							
8	0	1	0.09	30	5	4	0.78
9	0	0		31	5	3	0.69
10	0	0		32	7	5	1.03
11	0	0		33	37	21	5.00
12	0	0		34	29	32	5.25
13	0	1	0.09	35	8	4	1.03
14	0	0		36	6	3	0.78
15	0	0		37	5	4	0.69
16	1	0	0.09	38	1	0	0.09
17	3	0	0.26	39	0	1	0.09
18	1	6	0.60	*<Intermediate>*			
19	1	1	0.17	40	0	1	0.09
20	11	3	1.21	41	0	1	0.09
21	16	12	2.33	42	0	0	
22	9	8	1.55	43	0	0	
23	5	1	0.52	44	0	0	
24	5	2	0.60	45	0	1	0.09
25	8	4	1.03	46	0	1	0.09
26	145	79	19.29	47	1	0	0.09
27	227	189	35.75	48	0	0	
28	100	104	17.57	49	0	0	
29	12	20	2.76	50	0	1	0.09
				Total	648	513	100

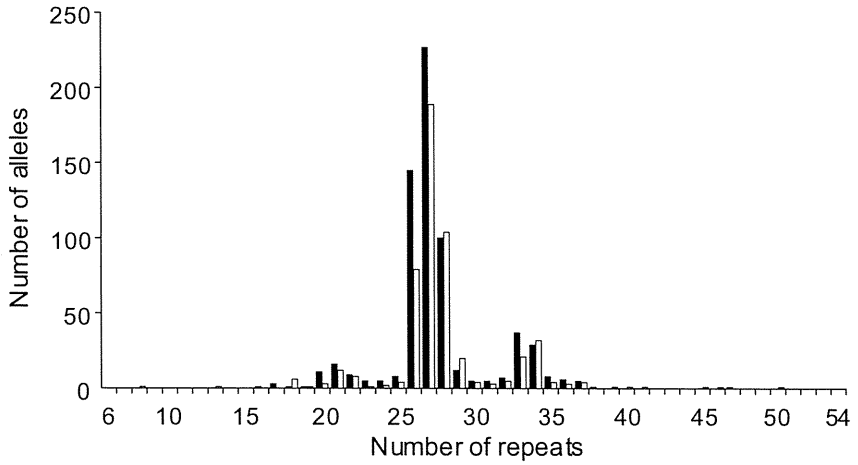

Fig. 1. Allele frequencies for CGG repeat in *FMR1* in 1161 normal Japanese population. Male and female were indicated by black and white column, respectively. See also Table 1.

Table 2
FMR1 allele frequencies in autistic patients and normal controls.

No. of *FMR1* CGG repeats		Autism ($n = 116$ alleles)		Controls ($n = 1161$ alleles)	
		Male ($n = 102$)	Female ($n = 14$)	Male ($n = 513$)	Female ($n = 648$)
6–39	(Normal)	102	14	508 (99%)	647 (99%)
40–54	(Intermediate)	0	0	5 (0.97%)	1 (0.15%)
55–200	(Premutation)	0	0	0	0
⩾200	(Full mutation)	0	0	0	0

be related to the different automated sequencer methodology and the PCR slippage. However, the difference was not simply associated with just the size but also the pattern, and thus the sample differences could have influenced the data.

In a previous screening of the mental retardation (MR) population in Japan, the prevalence of FXS ranged from 0.8% [15] to 2.4% [17] in males with MR. This result is slightly lower than that which has been reported for the Caucasian MR population rates, where it accounted for 2.6–8.7% among male patients with MR [29]. In contrast, the frequency of FXS in southern Taiwan [23] was 1.9% in the male MR populations, which is closer to that reported in Japan. These findings suggest that there is a difference in the prevalence between other Asian populations. However, since it is unlikely that all of the fragile X patients have been completely accounted for in Japan, it is important that a wide screening for FXS in the MR population be undertaken. Therefore, in order to more accurately study the prevalence of FXS, the frequency of the intermediate and premutation alleles within the normal population needs to be determined [5].

This is the first study that has focused on the prevalence of FXS by analyzing the intermediate and premutation alleles in the Japanese population. After screening 1161 X chromosomes from non-retarded healthy individuals, we found no carriers with the premutation allele. While the lack of any premutation allele has been confirmed in other reports in Japan [16,17], the number of collected samples in those studies was fewer than we collected in this study. Here, we estimated the frequencies of the intermediate allele (which ranged from 40 to 50 repeats) in normal Japanese subjects to be 1 in 103 males and 1 in 324 females. These frequencies were lower than the intermediate allele frequencies reported in the previous studies [10–13]. However, based on our findings, the prevalence of FXS subjects in Japan can be estimated to be 1 in about 10,000, which is lower than the predicted prevalence in Caucasian populations and in the subsets of Mediterranean and Pakistani populations (~1 in 4,000 males) [24,25]. Even so, it needs to be pointed out that the sample numbers used in our statistical analyses were relatively small and therefore, further analyses with sample numbers greater than 10,000 will need to be carried out in order to conclusively demonstrate the FXS prevalence in Japan. The structure of the CGG repeat may different in Japanese and the precise sequence study will be necessary.

We also performed an analysis that examined the length of the CGG repeats in Japanese autistic patients as a possible candidate locus for autism. The higher prevalence of autism that is seen in males versus females

suggests the possible involvement of the X chromosome. While some earlier studies have reported little or no association between FXS and autism [26,27], others have found a high association [28]. In this study, we did not find any significant difference in the distribution of the *FMR1* alleles nor did we find any premutation or intermediate alleles in any of the autism samples.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

A New Microdeletion Syndrome of 5q31.3 Characterized by Severe Developmental Delays, Distinctive Facial Features, and Delayed Myelination

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Chromosomal deletion including 5q31 is rare and only a few patients have been reported to date. We report here on the first two patients with a submicroscopic deletion of 5q31.3 identified by microarray-based comparative genomic hybridization. The common clinical features of both patients were marked hypotonia, feeding difficulty in infancy, severe developmental delay, and epileptic/nonepileptic encephalopathy associated with delayed myelination. Both patients also shared characteristic facial features, including narrow forehead, low-set and abnormal auricles, bilateral ptosis, anteverted nares, long philtrum, tented upper vermilion, edematous cheeks, and high palate. The deleted region contains clustered PCDHs, including and *PCDHG*, which are highly expressed in the brain where they function to guide neurons during brain development, neuronal differentiation, and synaptogenesis. The common deletion also contains neuregulin 2 (*NRG2*), a major gene for neurodevelopment. We suggest that 5q31.3 deletion is responsible for severe brain developmental delay and distinctive facial features, and that the common findings in these two patients representing a new microdeletion syndrome. We need further investigations to determine which genes are responsible for the patients' characteristic features. © 2011 Wiley-Liss, Inc.

Key words: microdeletion; 5q31.3; array-based comparative genomic hybridization (aCGH); developmental delay; protocadherin (PCDH); neuregulin 2 (*NRG2*)

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INTRODUCTION

Interstitial deletions of the long arm of chromosome 5 are rare, except in the 5q35.2q35.2 region that includes the 2-Mb *NSD1* locus which is associated with Sotos syndrome [Visser and Matsumoto, 2003]. Although patients with proximal deletions that encompass the 5q15 to q22 region experience mild developmental delays, those with distal deletions that encompass the 5q22 to q31 region are more severely handicapped, fail to thrive, and present with significant craniofacial dysmorphism and joint dislocations or contractures [Garcia-Minaur et al., 2005]. Furthermore, there are only a few reports of patients with deletions encompassing the 5q31.3 region [Felding and Kristoffersson, 1980; Kramer et al., 1999; Arens et al., 2004].

Recently, we encountered 2 patients with severe developmental delay and distinctive facial features. Microarray-based comparative genomic hybridization (aCGH) analyses identified a common microdeletion of 5q31 in both patients. Radiological examination yielded characteristic finding with delayed myelination in both patients. The details of these cases are discussed in this report. Data on the patients were deposited in the DECIPHER database (Database of Chromosomal Imbalances and Phenotype in Humans using Ensembl Resources, https://decipher.sanger.ac.uk), and the corresponding DECIPHER number is given.

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CLINICAL REPORTS

Patient 1 (DECIPHER #TWM253734)

A Japanese boy was born at 40 weeks 5 days gestation by caesarean when labor had failed to begin. He is the first child of a 30-year-old father and a 26-year-old mother at the time of his birth. His birth weight was 2,925 g (−0.3 SD), length 50 cm (+0.5 SD), and head circumference 35 cm (+1.3 SD). Postaxial polydactyly of the right hand was noted. Patent ductus arteriosus (PDA) and a small ventricular septal defect (VSD) were revealed by echocardiography; PDA was surgically treated when he was 52 days old, and the small VSD was observed but not treated. He showed failure to thrive due to severe hypotonia and feeding difficulty, and aspiration was suspected because of recurrent pneumonia. Tube feeding was initiated at 6 months of age. Although he had no epileptic episodes, his electroencephalography showed spike waves on the right side of the posterior and occipital regions during natural sleep. Auditory brainstem response revealed obscure III waves in both sides, and the threshold was 40 dB. His median nerve conductive velocity (NCV) showed a delay with 32.5 m/s (−2.1 SD) on the left and 30.5 m/s (−2.5 SD) on the right. His posterior tibial NCV was also revealed to be delayed with 27.6 m/s (−3.2 SD) on both sides. These findings indicated peripheral neuropathy.

At 18 months of age, he showed delayed growth and microcephaly with height 76.4 cm (−1.6 SD), weight 8.7 kg (−1.7 SD), and head circumference 42.8 cm (−3.0 SD). He showed distinctive features including narrow forehead, low-set and abnormal auricles, bilateral ptosis, epicanthic folds, depressed nasal bridge, anteverted nares, long philtrum, tented upper vermilion, edematous cheeks, and high palate (Fig. 1A). His developmental milestones were markedly delayed with no contact eye movements, no smile response, and no head control. Brain magnetic resonance imaging (MRI) revealed reduced volume of the cerebrum and severely delayed myelination (brain appearance was that of an 8-month-old child) in T2-weighted imaging (Fig. 2A). Chromosomal G-banding showed a normal male karyotype.

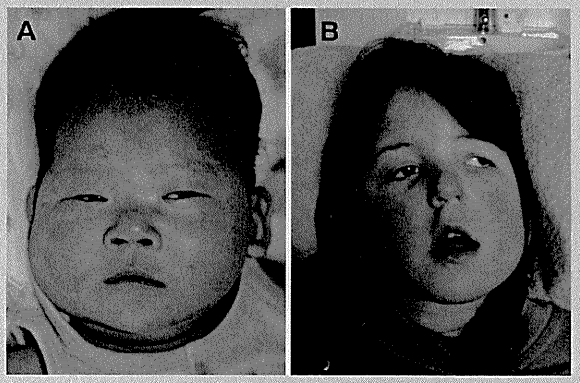

FIG. 1. Patient facial features. (A) Patient 1 at 18 months old. (B) Patient 2 at 6 years old.

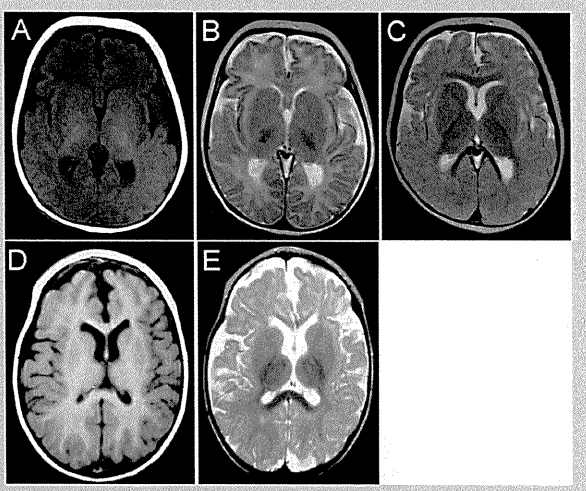

FIG. 2. Brain MRI findings with T1-weighted images (A, D) and T2-weighted images (B, C, E). Patient 1 at 4 months (A, B) and 18 months old (C). Patient 2 at her age of 4 years and 4 months old (D, E). Both patients showed reduced volume of the cerebrum associated with hypomyelination.

Patient 2 (DECIPHER #4681)

An 8-year-old French girl had no family history and no consanguinity in her parents. There was no complication during pregnancy. She was born with a birth weight of 3,700 g (+0.5 SD), a length of 52 cm (+1.0 SD), and a head circumference of 36 cm (+1.0 SD). Since early infancy, she showed feeding difficulties due to severe hypotonia. She had severe developmental delay with sitting at 11 months. Since the age of 12 months, she suffered epileptic seizures which were drug-resistant (hydrocortisone, clonazepam, topiramate, lamotrigine). Her epileptic status was diagnosed as Lennox–Gastaut syndrome.

She was of relatively small stature at a height of 121 cm (−1.5 SD), had a weight of 16.7 kg (−2.5 SD), and head circumference of 50 cm (−1.5 SD). She was not able to walk unassisted and was apraxic for speech. Her features were distinctive with narrow forehead, low-set ears, bilateral ptosis, downslanting palpebral fissures, anteverted nares, long philtrum, tented upper vermilion, edematous cheeks, and high palate (Fig. 1B). Strabismus was also noted. Brain MRI examination showed ventriculomegaly with reduced volume of the cerebrum, particularly in the frontoparietal regions, and marked hypomyelination (Fig. 2B). Conventional chromosome analysis showed a normal female karyotype.

MATERIALS AND METHODS

For further evaluation, microarray-based comparative genomic hybridization (aCGH) analyses, using Human Genome CGH Microarray 105A for Patient 1 and 44A for Patient 2 (Agilent Technologies, Santa Clara, CA), were performed according to the manufacturer's protocol, with genomic DNAs extracted from

peripheral blood samples. The identified aberrations were confirmed by fluorescence in situ hybridization (FISH) analyses, and both patients were also analyzed by FISH. Parental origin of the deletion in Patient 1 was determined using the microsatellite marker D5S1979 according to methods described elsewhere [Komoike et al., 2010]. Information regarding the primers used for the marker was obtained from the in-silico library (http://genome.ucsc.edu/).

RESULTS

Losses of genomic copies of 5q31.3 were identified in both patients. Patient 1 showed a 5.0-Mb deletion with molecular karyotyping as arr chr5q31.2q31.3(137,538,788–142,574,719)(hg18)x1 and Patient 2 showed a 2.6-Mb deletion with molecular karyotyping as arr chr5q31.3q31.3(139,117,448–141,682,547)(hg18)x1 (Fig. 3). FISH analyses with only one signal for the targeted probe confirmed the deletion (Fig. 4), and subsequent parental FISH analyses using the same probe showed no abnormality in their parents (data not shown), indicating de novo occurrence. Patient 1 shared the D5S1979 allele with his mother but not with his father (Fig. 4). This indicated that the deletion was paternally derived, and the final karyotype was ish del(5)(q31.2q31.3)(RP11-678N8x1) dn pat.

DISCUSSION

Both the patients in the present study showed an overlapping deletion of the region that included 5q31.3. The clinical features that were common for both patients were marked hypotonia, feeding difficulties in infancy, severe developmental delay, and epileptic/nonepileptic encephalopathy. Both patients also showed similar characteristic facial features, including a narrow forehead, low-set and abnormal auricles, bilateral ptosis, anteverted nares, long philtrum, tented vermilion of the upper lip, edematous cheeks, and high palate. Another characteristic finding was delayed myelination of the white matter, as identified by MRI examination. Thus, these findings are consistent, recognizable, and clinical features of 5q31.3 deletion.

To the best of our knowledge, five reports on patients with chromosome 5q31 deletions are available in the literature (Fig. 5). The first patient reported by Felding and Kristoffersson had manifestations similar to those of our patients [Felding and Kristoffersson, 1980]. Kramer et al. [1999] reported on a patient with 5q31q33 deletion whose condition was severely impaired; this patient showed congenital anomalies and died in the neonatal period. Arens et al. [2004] reported a patient with 5q22.1q31.3 deletion whose clinical findings included growth retardation, moderate psychomotor retardation, and mild facial dysmorphisms were similar to those of our patients. However, the severity of the developmental delay was milder than that of our patients, because she could walk without support and could speak a few words. These three patients were suspected to carry deletions of 5q31.3, but the deletion regions were ambiguous in conventional G-banding examination, and no neuroimaging test was available. We were thus unable to compare these patients with ours.

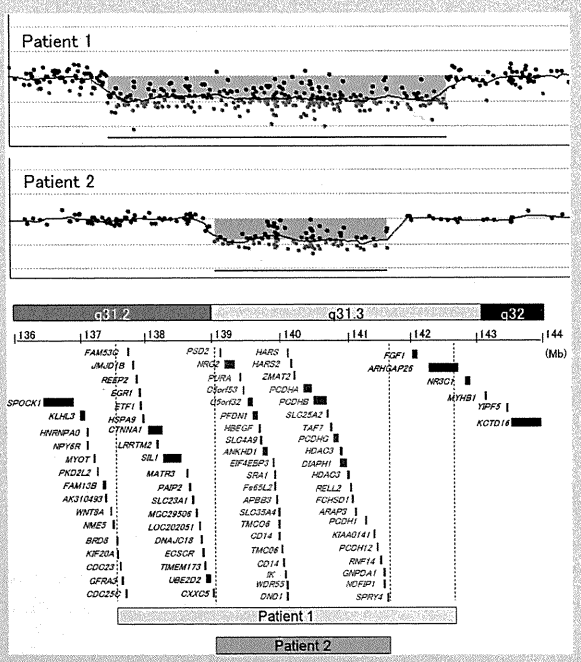

FIG. 3. The results of aCGH for chromosome 5. The aberrant areas of chromosome 5q31.2q31.3 are expanded in "Gene View" using CGH Analytics version 4.0 (Agilent) with the ADM-2 algorithm at a threshold of 6.0. The vertical axis indicates gain or loss of genome copy number and the horizontal axis indicates the physical position on chromosome 5. The aberrant areas are indicated by blue and red rectangles. Dots indicate the positions of the probes and green dots indicate $\log_2$ ratio under −0.5. Physical map corresponding to the highlighted area is depicted below the aCGH data. Rectangles indicate the positions of the UCSC genes (hg18). Colored rectangles indicate the genes included in the overlapped deletion region, and the genes showing high expression in the brain are indicated in red. Italic symbols indicate gene names.

Tzschach et al. [2006] reported on a patient with failure to thrive, psychomotor retardation, and mild facial dysmorphic features who carried a de novo deletion of 5q23.3q31.2, which did not overlap with those of our patients [Tzschach et al., 2006]. Mosca et al. [2007] reported a girl presenting with an abnormal cry, upslanting palpebral fissures, hypertelorism, anteverted nostrils, microretrognathia, growth retardation, and an adenoid cyst at the base of the tongue [Mosca et al., 2007]; the chromosomal deletion in this girl partially overlapped with that in Patient 1 of the present study, but the deletion did not involve the 5q31.3 band (Fig. 5).

In the present study, the common 2.6-Mb deletion region within the chromosomal band 5q31.3 is gene rich, containing 40 genes (UCSC Human genome browser, March 2006; http://genome.ucsc.edu/). The most intriguing finding is that the deleted region contained 5 genes classified as the protocadherin (PCDH) family which can be further divided into two main categories including clustered and non-clustered [Morishita and Yagi, 2007]. The clustered PCDHs including *PCDHA*, *PCDHB*, and *PCDHG*, which