Figure 1 (a) Profile of the copy-number ratio on chromosome X in the proband (III-1) detected with aCGH using an MCG X-tiling array. Each dot represents the test/reference value after normalization and log$_2$ transformation in each BAC clone, and arrows indicate duplications (ratio>0.4). The gray vertical lines represent the centromeric region for which no clones were available. Arrowheads indicate benign CNVs (Supplementary Table 1). (b) Three-generation genealogy of the studied family. Closed squares and circles and dotted circles indicate MR and carriers, respectively. The proband (III-1) indicated by an arrow was used for aCGH with the X-tiling array. Asterisks indicate persons having the duplications at Xq21.1 and Xq28. A slash indicates a death. (c) The proband (III-1) at 27 years showed hypertelorism, microcephaly and synophrys. (d) Brain MRI findings of the proband (III-1) at age 27. Coronal (left and middle) and (right) sagittal T1w sequences show cerebral atrophy, cerebellar atrophy and a thin corpus callosum. (e) Mapping of the duplications at Xq28 (left) and Xq21.1 (right) on the basis of the UCSC Genome Browser according to NCBI Build 36.1, March 2006, hg18 (http://www.genome.ucsc.edu/). A chromosome ideogram is presented. The track setting in the UCSC genome browser was set up to look for 'Base Positions', 'FISH Clones', 'BAC End Pairs', 'RefSeq Genes', 'sno/miRNA', 'Agilent Array' and 'Segmental Duplications'. Underlines below the BAC clone ID and oligonucleotide probe ID show a high (green bars) and normal (black bars) ratio detected with the MCG X-tiling array and Agilent array 244 K. Plus signs in the right panel indicate the duplication at Xq21.1 confirmed by quantitative genomic PCR. Duplicated regions in our case and the smallest region of overlap in reported cases[9] in the right panel are indicated with closed arrows. (f) In the proband (III-1) and the carrier mother (II-2), representative results of FISH using the clone RP11-42M11 at Xq21.1 (left) and the clone RP11-119A22 at Xq28 (right) showed separate green signals (arrowheads) and strong green signals (arrows), respectively. The red signals are of clone RP11-16H4 at Xp22.12 (left) RP11-13M9 at Xq13.2 (right) as a reference. Enlarged images of chromosome X are shown in the lower left insets in each panel. Similarly, in the affected proband's maternal cousin (III-3), representative results of FISH demonstrated separate red signals of the clone RP11-42M11 at Xq21.1 and strong green signals of the clone RP11-119A22 at Xq28. An enlarged image of chromosome X is shown in the lower right panel, indicating that the duplicated sequence at Xq21.1 inserted into the duplication at Xq28 together with the original Xq21.1 (arrowheads), whereas the duplicated sequence at Xq28 was inserted in close proximity (arrow). A full color version of this figure is available at the Journal of Human Genetics journal online.

by pneumonia frequently since 3 months after birth. No MRI analysis had been performed. His younger brother (III-4) died because of disseminated intravascular coagulation at the age of 29, and his other cousin (III-5) shows a similar clinical manifestation to the proband.

On the basis of the results of precise mapping with an oligonucleotide array (Agilent array 244K, Palo Alto, CA, USA; data not shown), these aberrations are as follows: arr Xq21.1 (76646979–76983735)×2, arr Xq28 (152847991–153262357)×2 (Figure 1e). Although some copy-number variants (CNVs) were detected in other regions simultaneously, all of them have been registered in the Database of Genomic Variants (http://projects.tcag.ca/variation/ assembly, March 2006, Supplementary Table 1) and in part in our CNV database (MCG CGH database, http://www.cghtmd.jp/CNVdatabase). Subsequent real-time quantitative genomic PCR (qPCR) using primer sets recognizing around dup(X)(q21.1) (Supplementary Table 2) narrowed down dup(X)(q21.1) to between positions 76646868 and 76973049, including all of ATRX and part of MAGT1 (Figure 1d). Fluorescence in situ hybridization (FISH) detected these duplications in the proband's unaffected mother (II-2) and his affected maternal male cousin (III-3) (Figures 1b and f), indicating maternally inherited duplications in these patients. In addition, the duplicated segment at Xq21.1 inserted into the duplicated region at Xq28, by contrast the segment at Xq28 was duplicated in tandem (Figure 1f). Our finding that the mother, a presumptive obligate carrier, had completely skewed X inactivation (dup(X):X=50:0) in a lymphoblastoid cell line , as shown by the androgen receptor X-inactivation assay described previously[5] and a late replication assay[6] with FISH (Supplementary Figure 1), supported our assumption that skewed X-chromosome inactivation appears to be characteristic of carriers of MECP2 duplication such as other reported cases.[3]

The two affected men showed severe MR, muscular hypotonia, recurrent respiratory infections and various other features characteristic of MECP2 duplication syndrome (Table 1). Moreover, they did not show short stature, hypoplastic genitalia and early life feeding issues, which were reported to be characteristic of MR in patients with duplications encompassing ATRX (Table 1).[9] The smallest region of overlap (SRO) of the reported ATRX duplication cases contains 11 genes, including ATRX and two miRNAs,[9] whereas the duplicated region of the present family includes only ATRX (Figure 1e), suggesting that genes other than ATRX within the SRO contribute to phenotypes observed in previously reported cases (Table 1).[9]

ATRX interacts with MECP2 in vitro and colocalizes at pericentromeric heterochromatin in mature neurons of the mouse brain.[10] Recently, it was reported that ATRX, MECP2 and cohesin cooperate to silence a subset of imprinted genes in the postnatal mouse brain.[11] Those experimental findings suggest that abnormally expressed ATRX with MECP2 through their simultaneous duplications may modify the phenotypes usually observed in MECP2 duplication syndrome. Although our patients showed neither notably different nor more severe phenotypes compared with reported patients with MECP2 duplication syndrome, the proband was found to have cerebellar atrophy by MRI (Figure 1e), which has never been reported before in MECP2 duplication syndrome.[1,3] It is possible that these phenotypes in the proband were modified through ATRX duplication in an additive or epistatic manner.

The mutations in ATRX give rise to changes in the pattern of methylation of several highly repeated sequences, including the ribosomal DNA (rDNA) arrays[12] and significantly altered mRNA expression in four ATRX targets (NME4, SLC7A5, RASA3 and GAS8) relative to normal controls.[13] Although a Southern blot hybridization method reported previously[12] showed no change in

Table 1 Phenotype comparisons between our cases and MECP2 duplication syndrome patients or patients with ATRX duplication

Phenotype	MECP2 duplication syndrome[3,7,8]	ATRX duplication cases[9]	Our Cases III-1	III-3
Mental retardation	118/119	11/11	+	+
Hypotonia	86/93	7/11	+	+
Absent speech	63/72	NA	+	+
Lack ambulation	20/71	NA	+	+
Recurrent infection	82/111	6/7	+	+
Breathing abnormalities	6/18	NA	−	+
Stereotyped hand movements	15/33	NA	+	+
Autistic features/autism	13/17	NA	+	+
Epilepsy	57/110	NA	+	+
GU abnormalities	29/67	7/10 (Hypoplastic genitalia)	−	+ (Bladder distention)
Death before 25 years	25/66	NA	−	−
Spasticity	42/71	NA	−	+
Ataxia	20/37	NA	+	+
GER	15/25	NA	−	+
Swallowing difficulty	23/45	NA	−	+
IPO or constipation	25/33	NA	−	−
IgA deficiency	4/10	NA	+	+
Short stature	NA	11/11	−	−
Early life feeding issues	NA	7/9	−	−
Failure to thrive	16/31	7/9	−	+
Broad thorax	NA	4/4	−	+
Pectus excavatum	NA	3/7	−	−
Short neck	NA	4/8	−	−
Simian crease	NA	4/5	−	−
Digital findings	22/52	6/7	−	−
Microcephaly	24/71	8/11	+	−
Hypertelorism	8/72	2/6	+	+
Epicanthal folds	4/72	6/8	−	−
Down-slanted palpebral fissures	NA	1/8	−	−
Ptosis	2/72	6/9	−	−
Flat nasal bridge	15/72	9/10	−	+
Down-turned corners of the mouth	NA	8/10	−	−
High-arched palate	3/72	4/4	−	−
Micro/retrognathia	NA	4/7	−	−
Low set ears	NA	4/10	−	−
Simple ears	NA	2/10	−	−
Cryptorchidism	2/4	9/10	+	−
Impaired social interaction	NA	5/6	+	+

Abbreviations: ATR-X, the causative gene for X-linked alpha thalassemia/mental retardation; GER, gastroesophageal reflux; GU, genito-urinary system; IPO, intestinal pseudo-obstruction; MECP2, methyl CpG binding protein 2; NA, not available.

the pattern of methylation at rDNA arrays compared with normal controls (Figure 2a), quantitative RT-PCR revealed that the expression of ATRX was upregulated in the present cases. Although SLC7A5 expression showed no previous change compared with that in the healthy control (Figure 2b) and the expression of GAS8 was too low for quantitative RT-PCR (data not shown), the expression of NME4 and RASA3 was similar to that in the patients with ATRX mutations. The alteration to the expression may be influenced by MECP2 duplication or additive/epistatic effect between ATRX and MECP2 duplication.
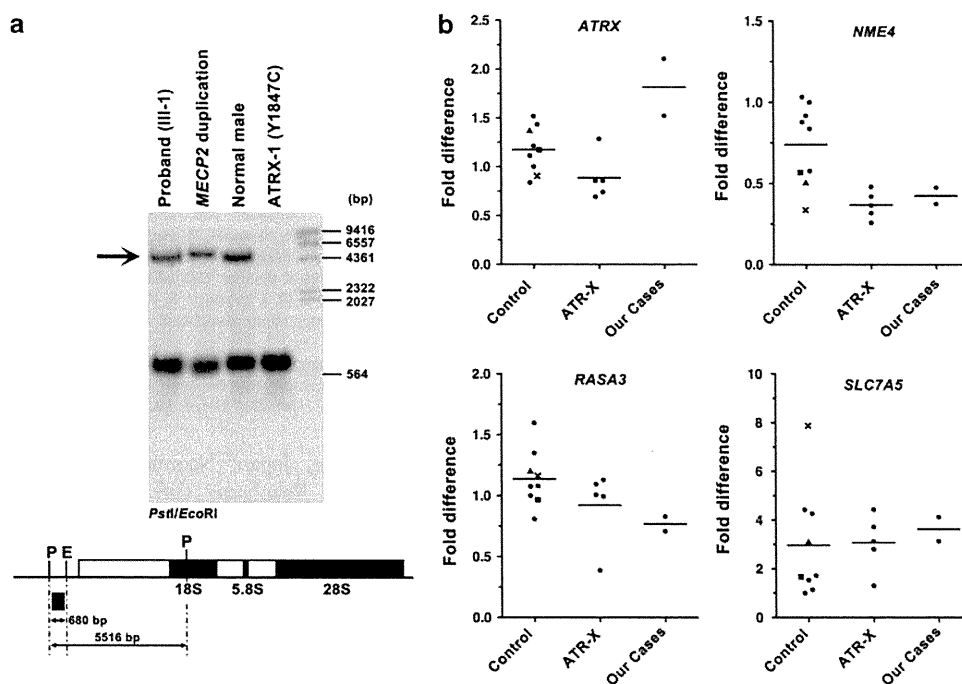
Figure 2 (a) Investigation of the methylation pattern of human rDNA repeats using Southern blotting. Genomic DNA from the lymphoblastoid cell line of our proband (III-1) with the *ATRX* duplication and the *MECP2* duplication, an MR male patient with *MECP2* duplication, an unrelated normal male, and an ATR-X patients with a missense mutation resulting in Y1847C in ATRX (Supplementary Table 3). DNA samples were digested with *Pst*I followed by the methylation-sensitive enzyme *Eco*RI. Hybridization is shown for probes corresponding to the region between restriction sites of the two enzymes in the 3′ end of the non-transcribed spacer. The methylated, uncut band is indicated (arrow). A restriction map of part of the rDNA repeat unit shown with the 18S, 5.8S and 28S genes in order and transcribed spacer as filled and open boxes, respectively, represents the sites for *Pst*I (P) and *Eco*RI (E). A black bar indicates the probe for the Southern hybridization. The size of the DNA segment resulting from the restriction enzymes is represented by closed arrows. (b) Real-time quantitative RT-PCR analysis of the mRNA expression of *ATRX* and three *ATRX* target genes (*NME4*, *RASA3* and *SLC7A5*) but not *GAS8*, the expression of which was too low to be estimated, in lymphoblastoid cells of our two patients, ATR-X patients whose *ATRX* mutations were identified through routine screening in a set of known XLMR genes by the Japanese Mental Retardation Consortium (unpublished data, *n*=5; Supplementary Table 3) and controls, including six healthy samples, the proband's parents, and a patient and a carrier with the *MECP2* duplication.[2] All the subjects provided written informed consent for the use of their phenotypic and genetic data. The proband's carrier mother, the patient and the carrier with the *MECP2* duplication are represented by a cross, triangle and square, respectively, in the control column. Data show the average values for fold differences relative to a normal male. Black bars represent mean values of each group.

The result of FISH suggests that *ATRX* duplication and *MECP2* duplication were occurred simultaneously resulting in complex genomic rearrangement. The proximal breakpoint of dup(X)(q21.1) and distal breakpoint of dup(X)(q28) were located on segmental duplications (Figure 1e) and the duplicated sequence at Xq21.1 existed near dup(X)(q28) (Figure 1f). Fork Stalling and Template Switching (FoSTeS) has been proposed as a replication-based mechanism that produces nonrecurrent rearrangements potentially facilitated by the presence of segmental duplications.[14] Previous reports suggested that complex genomic rearrangements at Xq28 such as an embedded triplicated segment and stretches of non-duplicated sequence within dup(X)(q28) were probably mediated by FoSTeS,[7,15] and a particular genomic architecture, especially low copy repeats at distal breakpoints of dup(X)(q28), may render the *MECP2* region unstable. Thus, the dup(X)(q28) and dup(X)(q21.1) detected in our patients might be generated simultaneously by FoSTeS or other mechanism in a segmental duplication-dependent manner, suggesting the structural analysis of the entire X chromosome in patients with dup(X)(q28) to be important for understanding their correct clinical condition and providing appropriate education.

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APPENDIX

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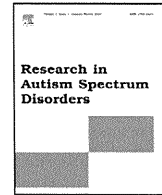
Parental age and assisted reproductive technology in autism spectrum disorders, attention deficit hyperactivity disorder, and Tourette syndrome in a Japanese population

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ABSTRACT

We investigated whether advanced parental age and assisted reproductive technology (ART) are risk factors in autism spectrum disorders (ASDs), attention deficit hyperactivity disorder (ADHD), and Tourette syndrome (TS). Clinical charts of Japanese outpatients with ASD ($n = 552$), ADHD ($n = 87$), and TS ($n = 123$) were reviewed. Parental age of individuals with ASD, ADHD, or TS was compared with parental age in the general population (GP) of Tokyo after adjusting for year of birth. Paternal and maternal ages were significantly higher in persons with ASD and ADHD, but not those with TS. In final steps of stepwise logistic regression analysis, both maternal and paternal age were associated with ASD after controlling for the other parent's age, gender, and birth order. In cases where the presence or absence of ART could be ascertained (ASD $n = 467$; ADHD $n = 64$; TS $n = 83$), the rate of ART in cases of persons with ASD (4.5%) was 1.8 times the frequency expected in the GP, while ART was not present in cases of persons with ADHD and TS. These preliminary results remain tentative pending replication with larger, community-based samples.

1. Introduction

The etiology of autism spectrum disorders (ASDs) is not well understood. Recent studies suggest that *de novo* mutations (Marshall et al., 2008; O'Roak et al., 2011; Pinto et al., 2010; Sebat et al., 2007) and epimutations (Grafodatskaya, Chung, Szatmari, & Weksberg, 2010) in the genome play a role in ASD. Advanced parental age at delivery and assisted reproductive technology (ART) may be mediating factors in this process (Bonduelle et al., 2002; Manipalviratn, DeCherney, & Segars, 2009; Rivera et al., 2008). An association between advanced parental age and ASD has been reported mainly in studies from the United

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States (Croen, Najjar, Fireman, & Grether, 2007; Durkin et al., 2008; Grether, Anderson, Croen, Smith, & Windham, 2009; King, Fountain, Dakhlallah, & Bearman, 2009; Shelton, Tancredi, & Hertz-Picciotto, 2010), Europe (Hultman, Sandin, Levine, Lichtenstein, & Reichenberg, in press), and Israel (Reichenberg et al., 2006). Many studies (Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009; Hultman et al., in press; King et al., 2009; Reichenberg et al., 2006; Shelton et al., 2010) have reported similar significant associations between the occurrence of ASD and advanced parental age. However, several questions remain.

The first question is whether advanced parental age directly affects the development of ASD symptoms (i.e., difficulties with social situations and/or reciprocal communication with people), or whether it affects intellectual or general aspects of child development that could be associated with the risk of an ASD. A previous study reported an association between advanced paternal age and lowered intelligence quotient (IQ) in the general population (Saha et al., 2009), but it also found an association between advanced maternal age and higher IQ. Most previous studies have not separately investigated parental age and ASD according to whether or not the child displayed some degree of mental retardation (MR). One exception may be Tsuchiya et al. (2008), who identified advanced paternal age in individuals with ASD and IQ > 70, but the use of a control group unmatched for sex and age means that caution must be used when interpreting results: mean parental age in cases where ASD was present was five years less than mean parental age in the control group. These results could be affected by the fact that parental age in Japan has increased over the last few decades. Thus, the issue remains to be clarified.

If advanced parental age affects general brain development, it is reasonable to hypothesize that this impact might also be observed in other developmental disorders, such as attention deficit hyperactivity disorder (ADHD). Thus far, however, only one study has investigated this issue in relation to ADHD, and it found no association with parental age (Gabis, Raz, & Kesner-Baruch, 2010).

The second question is whether the association of advanced parental age with ASD is confined to cases of advanced paternal age, advanced maternal age, or both. Two recent large-scale population-based studies conducted in Europe and Israel reported an association between ASD and paternal age, but not maternal age (Hultman et al., in press; Reichenberg et al., 2006). In the United States, one study reported an association with maternal age (King et al., 2009), while four studies reported associations with both paternal and maternal ages (Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009; Shelton et al., 2010). Thus, the issue remains to be clarified.

In Asian populations, in contrast to Western populations, few studies have investigated the association between ASD and parental age. Case–control studies in Japan and China found an association with paternal age (Tsuchiya et al., 2008; Zhang et al., 2010). Another study (Koyama, Miyake, & Kurita, 2007) found that both paternal and maternal ages were elevated in cases of persons with ASD, compared with place-matched general population data.

The present study also attempted to explore whether an association exists between assisted reproductive technology (ART) and ASD. ART includes in vitro fertilization with trans-cervical embryo transfer (IVF) and intracytoplasmic sperm injection (ICSI). The number of children born using ART has been sharply increasing in the past decades in Japan and other developed countries. The first IVF child was reported in the UK in 1978 (Steptoe & Edwards, 1978). The first ICSI child was born in Belgium in 1992 (Palermo, Joris, Devroey, & Van Steirteghem, 1992). In Japan, the first IVF child was reported in 1983, and the first ICSI child in 1994 (Yanagida et al., 1994); in 2006, 1.8% of all Japanese newborns were by ART (Japan Society of Obstetrics and Gynecology, 2008).

ART may have the potential to affect the genetic and/or epigenetic structure of the genome in gametes, fertilized eggs, and embryos through its procedures and unnatural selection of germ cells. However, a limited number of studies have explored the impact of ART on developmental disorders, including ASD. Using birth registry data from Finland, Klemetti, Sevon, Gissler, and Hemminki (2006) compared cases of 4500 children born via IVF and 27,000 born via natural conception (NC), and found that ART was associated with a broad range of psychiatric conditions, including disorders of psychological, behavioral and emotional development. Knoester et al. (2007) followed children born after ICSI, IVF, and NC. After diagnosing these children at the age of 5–8 years based on parental report, they identified ASDs in three of 87 children born via ICSI; none in 85 children born via IVF; and one in the 81 children born via NC. These results may be inconclusive due to the small sample size.

Using the Danish national registry (Maimburg & Vaeth, 2007) compared the frequency of assisted conception (AC) in persons with autism and age-, sex- and birth place-matched healthy controls. The frequency of AC was not higher in autism (2.3%; $n = 461$) than in controls (5.4%; $n = 461$). Another study using the Danish registry found the incidence of ASD to be 0.68% in children born via AC ($n = 33,000$) and 0.61% in children born via NC ($n = 556,000$) (Hvidtjorn et al., 2010). The effect of AC was not found to be significant after adjusting for confounders. These studies did not separately analyze ART and other AC (ovulation induction and/or intrauterine insemination). Thus, there are few studies of the impact of ART on developmental disorders, including ASD, and those that do exist tend to be inadequate.

In an attempt to explore these questions, we conducted a chart review study of persons diagnosed with ASD and other developmental disorders, including ADHD and Tourette syndrome (TS). Parental age and frequency of ART were investigated for each of these three disorders.

2. Methods

2.1. Setting and procedures

We conducted a retrospective chart review of persons diagnosed with ASD, ADHD, and TS who first visited the child psychiatry outpatient clinic of the University of Tokyo Hospital, from April 2006 to March 2009. These disorders were the

three most frequent disorders at the outpatient clinic during the period. Diagnosis was made according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria (APA, 2000) by child psychiatrists. Many patients were referred from rehabilitation facilities for children or private clinics.

Most (>90%) of the patients lived in the Tokyo Metropolis area, which has a population of approximately 13 million people. Generally, 70–90% of the cost for the medical care is covered by public health insurance in Japan.

Our review of clinical charts allowed the identification of 814 persons diagnosed with ASD, ADHD, or TS. We subsequently excluded cases of persons born before 1975 (n = 33), those who were not Japanese (n = 10), or those with nervous or systemic diseases (n = 4; three persons in the ASD group (postoperative brain tumor, postencephalopathy, and neurofibromatosis type 1) and one in the ADHD group (Turner Syndrome)). Five pairs of co-affected twins (four cases of monozygotic twins and one case of dizygotic twins) were found in the ASD group (there were none in either the ADHD or TS groups). For these cases, the co-twin with the more severe degree of disorder was included for each twin pair. This resulted in a pool of 552 persons diagnosed with ASD, 87 with ADHD, and 123 with TS. Of these individuals, we were able to determine whether or not ART had been used in 467 of the cases of ASD, 64 of the cases of ADHD, and 83 of the cases of TS.

2.2. Data analysis

Clinical charts were reviewed by two of the authors and the demographic and clinical information was studied, including sex, birth year, ages of mother and father at the delivery, education levels of the parents, use/no use of ART for the individuals birth, concurrence of MR, singleton/multiple birth, birth weight and gestational age at the delivery.

The information of ART was obtained from a questionnaire filled in by the parents at the visits of the clinic and from the clinical record written by the attending doctors or psychologists. IQ was measured using a Japanese version of Wechsler test (WISC-III (n = 313), WAIS-III (n = 68) or WPPSI (n = 3), according to the age of the subjects) or a Japanese version of Binet test (Tanaka–Binet test, n = 98).

This study was approved by the Ethics Committee of the Faculty of Medicine at the University of Tokyo.

General population (GP) data of Tokyo Metropolis was employed as control data for the parental ages and other variables (Ministry of Health, Labour and Welfare 2008). Average maternal age of the GP in the birth year was used as the "control maternal age" for each subject. Average of the control maternal age was then calculated in each disorder (ASD, ADHD and TS). Average of the control paternal age was calculated by the same method. The maternal and paternal ages in each disorder was compared with the control ages using one-sample t-test.

In ASD, stepwise logistic regression analysis was conducted to estimate the effect of parental age after controlling the other parent's age, gender, singleton or multiple birth, gestational age, birth weight, and birth order as potential confounding factors. The GP data of Tokyo in 2002 was used as the control, because the mode birth year in ASD subjects was 2002.

All statistical analyses were conducted using SPSS version 17.0 (SPSS, Chicago, IL, US).

3. Results

3.1. Demographic features

We analyzed a total of 762 persons with either ASD (n = 552), ADHD (n = 87), or TS (n = 123). In the 552 ASD cases, 205 (37%) were diagnosed with autistic disorder (163 males and 42 females), and the rest were diagnosed with Asperger disorder or Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) (n = 347; 264 males and 83 females). Age at the time of first visit to the clinic was 9.8 ± 6.6 in the ASD group, 15.4 ± 6.3 in the ADHD group, and 11.4 ± 6.4 in TS group (mean ± SD; years). The mode (range) of birth years was 2002 (1975–2006) in the ASD group, 2001 (1975–2003) in the ADHD group, and 1996 (1975–2003) in the TS group.

Demographic features of the individuals with these three disorders are summarized in Table 1. For reference, population data for all children born in 2002 in Japan (2002 is the mode birth year for the ASD group). No significant difference was found in either paternal or maternal educational level among the three disorders.

3.2. Parental age

Table 2 summarizes mean parental ages at delivery in persons studied and in the general population of Tokyo. The mean ages in general population data were adjusted for the distribution of birth years in the individuals studied. Compared with the general population of Tokyo, mean paternal age and maternal age were significantly higher in persons with ASD (34.2 ± 5.8 and 31.6 ± 4.7 years, respectively) and ADHD (35.1 ± 6.2 and 32.2 ± 4.9 years, respectively). No difference was observed in persons with TS.

Among persons with ASD, paternal and maternal ages were significantly higher in those with autistic disorder than those with Asperger disorder or PDD-NOS (35.2 ± 6.1 vs. 33.6 ± 5.5 years for paternal age, t = 2.58, df = 368, p = 0.01; and 32.3 ± 4.5 vs. 31.2 ± 4.7 years for maternal age; t = 2.19, df = 381, p = 0.03, t-test, respectively). Maternal age was significantly higher in persons with autistic disorder and Asperger disorder or PDD-NOS than in the general population of Tokyo. While paternal age was significantly higher in persons with autistic disorder than in the general population of Tokyo, no difference was observed for persons with Asperger disorder or PDD-NOS.

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Table 1
Characteristics of Japanese patients with ASD, ADHD, and TS, and general population data of Tokyo in 2002.

	ASD		ADHD		TS		General population data of Tokyo in 2002	
	No.	%	No.	%	No.	%	No.	%
Gender								
Male	427	77%	74	85%	82	67%	51,619	52%
Female	125	23%	13	15%	41	33%	48,499	48%
IQ ≤ 70	146	32%	1	1%	5	14%	NA	
IQ > 70	310	68%	69	99%	31	86%	NA	
Singleton	534	97%	84	97%	123	100%	97,843	98%
Multiple birth	18	3%	3	3%	0	0%	2275	2%
Gestational age, weeks								
<37	36	7%	3	4%	5	5%	62,289	5%
≥37	472	93%	72	96%	101	95%	1,091,074	95%
Birth weight for gestational age								
<2500 g	51	10%	5	6%	1	1%	9162	9%
≥2500 g	480	90%	75	94%	115	99%	90,935	91%
Birth order								
1st	360	67%	56	70%	78	65%	53,947	54%
(Single child only)	(168)	31%	(29)	36%	(31)	26%	NA	
2nd	137	26%	20	25%	34	28%	34,594	35%
≥3rd	37	7%	4	5%	8	7%	11,577	12%
Paternal education								
Less than high school	17	3%	4	5%	4	3%	NA	
High school graduate	113	22%	19	25%	34	29%	NA	
Some college	323	64%	50	65%	70	60%	NA	
≥4-year college graduate (postgraduate)	55	11%	4	5%	8	7%	NA	
Maternal education								
Less than high school	11	2%	2	3%	4	3%	NA	
High school graduate	150	29%	26	33%	44	38%	NA	
Some college	345	67%	52	65%	69	59%	NA	
≥4-year college graduate (postgraduate)	12	2%	0	0%	0	0%	NA	
Paternal age at delivery, years								
<30	66	18%	6	13%	24	37%	471,069	40%
30–34	150	41%	21	47%	27	42%	401,772	34%
35–39	104	28%	7	16%	10	15%	216,070	18%
≥40	50	14%	11	24%	4	6%	86,088	7%
Maternal age at delivery, years								
<30	133	36%	11	24%	33	51%	685,322	58%
30–34	157	42%	18	40%	25	38%	371,265	32%
35–39	73	20%	16	36%	6	9%	105,838	9%
≥40	20	5%	1	2%	2	3%	12,574	1%

Abbreviations: ASD, autism spectrum disorder; ADHD, attention deficit hyperactivity disorder; TS, Tourette syndrome; IQ, intelligence quotient; NA, not available.

No difference in paternal and maternal age was observed between males and females with ASD.

We also examined differences in parental age in persons with ASD and with/without MR. No significant difference was observed in either paternal or maternal age in persons with ASD and with/without MR. There was no significant correlation between full IQ and either paternal or maternal age ($r = -0.07$, $p = 0.25$, and $r = -0.06$, $p = 0.32$, respectively, Spearman correlation). Parental ages in persons with ASD and with/without MR were significantly higher than those in the general population of Tokyo.

We conducted stepwise logistic regression analysis in the ASD group using 2002 population data for Tokyo as a control, in order to test the effect of parental age after adjusting for the other parent's age, gender, singleton or multiple birth, gestational age, birth weight, and birth order as potential confounding factors (Table 3). In the final steps of stepwise method, three variables were selected: the other parent's age, gender, and birth order. The adjusted ORs (95% confidence interval) for the ASD group were 1.8 (1.3–2.4), 2.1 (1.4–3.0), and 2.0 (1.3–3.1) in cases where fathers were aged 30–34, 35–39, and ≥40 years, respectively, when compared with those aged <30. Regarding maternal age, the adjusted OR was 2.9 (1.7–5.1) in cases where mothers were aged ≥40 years compared with those <30, but there was no significance when maternal age was under 40. In contrast, the results for birth order suggested that the decline in ASD risk associated with increasing birth order.

3.3. Assisted reproductive technology (ART)

Information about the use of ART was available for 467 persons diagnosed with ASD, 64 with ADHD, and 83 with TS born after 1988. No person identified was born using ART before 1989. ART was used in 21 cases where the person was

Table 2

Mean paternal and maternal ages at delivery in persons with ASD, ADHD, and TS, and general population data in Tokyo after adjustment for birth year.

	Paternal age at birth, years ± SD		Maternal age at birth, years ± SD	
		General population data in Tokyo, after adjustment for birth year		General population data in Tokyo, after adjustment for birth year
ASD	34.2 ± 5.8 (n = 370)	33.0	31.6 ± 4.7 (n = 383)	30.4
ADHD	35.1 ± 6.2 (n = 45)	32.8	32.2 ± 4.9 (n = 46)	30.2
TS	31.9 ± 5.0 (n = 65)	32.7	29.8 ± 4.5 (n = 66)	29.6
Autistic disorder	35.2 ± 6.1 (n = 135)	33.1	32.3 ± 4.5 (n = 139)	30.5
ASP or PDD-NOS	33.6 ± 5.5 (n = 235)	33.0	31.2 ± 4.7 (n = 244)	30.3
Male ASD	34.0 ± 5.4 (n = 276)	NA	31.6 ± 4.6 (n = 285)	NA
Female ASD	34.8 ± 6.8 (n = 94)	NA	31.7 ± 4.9 (n = 98)	NA
ASD with MR	34.6 ± 6.2 (n = 78)	33.0	31.8 ± 4.4 (n = 80)	30.3
ASD without MR	33.8 ± 5.7 (n = 208)	32.9	31.4 ± 4.9 (n = 215)	30.3

Abbreviations: ASD, autism spectrum disorder; ADHD, attention deficit hyperactivity disorder; TS, Tourette syndrome; ASP, Asperger disorder; PDD-NOS, pervasive developmental disorder not otherwise specified; MR, mental retardation; SD, standard deviation; NA, not available.

Table 3

Distribution of ASD group and general population data of Tokyo in 2002 by parental age categories and other independent risk factors selected at final steps of stepwise logistic regression analysis, and unadjusted and adjusted odds ratios with 95% confidence intervals.

	ASD group		General population data of Tokyo in 2002		Unadjusted OR	95% CI		Adjusted OR[a]	95% CI		p value
	No.	%	No.	%							
Paternal age at delivery, years											
<30	60	17%	28,160	29%	1.0	Reference		1.0	Reference		
30–34	138	40%	36,921	38%	1.8	1.3	2.4	1.8	1.3	2.6	<0.001
35–39	98	28%	22,754	23%	2.0	1.5	2.8	2.1	1.4	3.0	<0.001
≥40	49	14%	10,270	10%	2.2	1.5	3.3	2.0	1.3	3.1	0.003
Maternal age at delivery, years											
<30	113	33%	40,845	42%	1.0	Reference		1.0	Reference		
30–34	143	41%	39,536	40%	1.3	1.0	1.7	1.1	0.9	1.5	0.369
35–39	70	20%	15,617	16%	1.6	1.2	2.2	1.4	1.0	2.0	0.058
≥40	19	6%	2107	2%	3.3	2.0	5.3	2.9	1.7	5.0	<0.001
Gender											
Male	256	74%	50,600	52%	2.7	2.1	3.4	2.7	2.1	3.5	<0.001
Female	89	26%	47,505	48%	1.0	Reference		1.0	Reference		
Birth order											
1st	229	66%	52,879	54%	1.0	Reference		1.0	Reference		
2nd	89	26%	34,246	35%	0.6	0.5	0.8	0.5	0.4	0.7	<0.001
≥3rd	27	8%	10,980	11%	0.6	0.4	0.8	0.4	0.3	0.6	<0.001

The variables as potential confounding factors, the other parent's age, gender, singleton or multiple birth, gestational age, birth weight, and birth order, were submitted to a stepwise logistic regression model.
Abbreviations: ASD, autism spectrum disorder; CI, confidence interval; OR, odds ratio.
[a] Adjusted for the other parent's age, gender, and birth order.

diagnosed with ASD, but was not used in cases where the person was diagnosed with ADHD or TS. The characteristics of the 21 persons born using ART are summarized in Table 4. The observed number (n = 21) was 1.8 times the expected number (n = 11.7), which was estimated according to population data in Tokyo (Japan Society of Obstetrics and Gynecology, personal communication, 2011), after adjusting for the distribution of birth years in cases studied.

4. Discussion

4.1. Parental age at delivery

We conducted a retrospective chart review to investigate parental age at delivery and the use of ART in persons diagnosed with ASD, ADHD and TS. Parental age was elevated in cases of ASD in our Japanese population. We also found advanced

Table 4
Number and characteristics of births using assisted reproductive technology in persons with ASD born after 1988.

	ASD ($n = 467$)
Number of births via assisted reproductive technology	21 (4.5%)
Male/female	16/5
Birth year (mode (range))	2003 (1989–2006)
Multiple birth/singleton	9/11
Autistic disorder/ASP or PDD-NOS	13/8
ASD with MR/without MR	6/7
Paternal age, years ± SD	38.9 ± 4.2
Maternal age, years ± SD	37.8 ± 4.0

Abbreviations: ASD, autism spectrum disorder; ASP, Asperger disorder; PDD-NOS, pervasive developmental disorder not otherwise specified; MR, mental retardation; SD, standard deviation.

parental age in cases of ADHD, but not in TS, compared with GP data matched for birth place and year. These results suggest that elevated parental age is associated on a significant level not only in ASD, but in some other child-onset disorders, such as ADHD, as well. It is not clear why the association was not significant in TS. The nature of disturbance in brain development might be different in TS compared with ASD and ADHD, or it could be due to methodological limitations of this study, including the method of case selection and sample size.

No parental age difference was observed in those persons with ASD and with/without MR. This suggests that the effect of parental age on the development of ASD may not be specifically through an impact on intellectual development.

This raises the question of whether parental age affects specific domains of ASD symptoms, such as disorders in social interaction, communication, and restricted patterns of interested behavior, or whether it affects the development of more general brain functions. We did not evaluate specific symptoms of ASD in the present study. However, advanced parental age was also observed in persons with ADHD, not only in those with ASD. In addition, several studies have observed advanced parental age in schizophrenia (Torrey et al., 2009), which is also considered to be related to early neurodevelopment disturbances (Rapoport, Addington, Frangou, & Psych, 2005). These findings indicate that the effect of parental age might be on general aspects of brain development, not on a specific brain function. This may in turn later affect several aspects of brain function related to social interaction and cognition. This speculation would be consistent with the absence of advanced parental age observed in persons with TS, as the symptom spectrum of TS may be more confined to specific disturbances such as tics, rather than ASD or ADHD (APA, 2000).

Whether paternal age, maternal age, or both have an effect on the development of ASD has been controversial in previous studies (Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009; King et al., 2009; Koyama et al., 2007; Reichenberg et al., 2006; Shelton et al., 2010; Tsuchiya et al., 2008; Zhang et al., 2010). We conducted logistic regression analysis in the group with ASD, with place-matched GP data as a control. Results supported the hypothesis that both paternal and maternal ages tend to be elevated in ASD, after adjusting for the age of the other parent (Table 3). The magnitude of the effect of paternal age (OR = 1.8–2.1 for fathers of age 30 years or over) might be comparable to results of previous studies that observed an association with ages of both parents ((Croen et al., 2007; Durkin et al., 2008; Grether et al., 2009); OR = 1.2–1.4). Similar to these studies, the association with maternal age was significant when age was ≥40 years.

This study suggested that the risk of ASD was highest for firstborn children and declined with increasing birth order, similar to two previous studies (Croen et al., 2007; Durkin et al., 2008). A possible explanation for the birth order effect is that parents having a child with ASD may tend to rarely have subsequent children because of the demands of parenting a child with a disability or concerns about genetic susceptibility (Jones & Szatmari, 1988).

4.2. Assisted reproductive technology (ART)

We performed a preliminary study of the frequency of the three disorders (ASD, ADHD and TS) in cases where ART (IVF and ICSI) was used. The hypothesis behind this is that ART may be associated with the risk of developmental disorders, possibly through its impact on genomic and epigenomic structure. An increase of *de novo* microscopic chromosomal anomalies has been observed in children born via ICSI (Bonduelle et al., 2002). The incidence of Beckwith–Wiedemann syndrome and Angelman syndrome due to epigenetic anomalies was also greater in children born using ART (Manipalviratn et al., 2009). Few studies have explored the association between ART and child-onset developmental disorders.

In the present study, cases of ASD had a rate of ART use of 4.5% (21 out of 466 cases), which was 1.8 times the expected frequency, based on GP data for Tokyo (2.5%) (Japan Society of Obstetrics and Gynecology, personal communication, 2011). In contrast, no cases of ART use were found in persons diagnosed with ADHD or TS.

However, when only the singleton individuals were examined, no difference was observed in the frequency of ART use between persons with ASD and GP data of Tokyo. The rate of multiple births was significantly higher in ASD cases where ART was used compared to where it was not. Thus, our preliminary study did not provide evidence for an increased rate of ASD in cases where ART was used. These results should be interpreted carefully because parental age was significantly higher in ASD cases where ART was used compared to where it was not.

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4.3. Strengths and limitations

Individuals in the present study were all Japanese (at least both parents were Japanese), which means that the sample tended toward ethnic homogeneity. This may be a strength compared with studies in other populations.

Limitations must also be acknowledged. First, general population data of Tokyo, taken from national statistics, were used as control data in this study. We adjusted the data according to the respective distribution of birth years in persons with ASD, ADHD, and TS. However, results were not adjusted for the sex of children and their socioeconomic status, including the education level of the parents. This could affect the results.

In contrast, the education levels of parents did not appear to be significantly different in persons with any of the three disorders. Therefore, it may not have an impact on any comparison of the disorders. Second, the general population may include individuals with ASD, ADHD, and TS. The respective prevalence of ASD and ADHD in Japan has been reported as being 1.2% (Honda, Shimizu, & Rutter, 2005) and 7.7% (Kanbayashi, Nakata, Fujii, Kita, & Wada, 1994). Epidemiological studies of persons with TS have not been conducted in Japan, but in other countries the prevalence has been estimated at around 1% (Robertson, 2008). With regard to persons with ASD and TS, the impact of having similarly affected persons in the general population data might be small on the present results, considering their low prevalence. In persons with ADHD, however, it could have some impact results.

Third, data was obtained by reviewing clinical charts, and some information was missing. This decreased the number of the persons who could be included in the statistical analyses. Fourth, sample size was limited, especially in relation to ADHD and TS. Finally, diagnoses were not made using structured interviews/observations, such as the Autism Diagnosis Interview-Revised (ADI-R) (Le Couteur, Rutter, & Le Couteur, 2003) and the Autism Diagnosis Observation Schedule (ADOS) (Lord, Rutter, DiLavore, & Risi, 1999), because those diagnostic tools were not available in Japan during the study period. Although diagnoses were made by skilled child psychiatrists (those with >10 years of experience), this could also be a weakness of the present study.

In summary, the present study observed elevations in both paternal and maternal age in persons with ASD and ADHD, but not in those with TS. In the case of ASD, the effect of parental age might not be significantly different between persons with and those without MR. In a preliminarily analysis, the frequency of ART use appeared to be higher in cases of persons with ASD than in the general population.

Acknowledgements

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ORIGINAL ARTICLE

Association between oxytocin receptor gene polymorphisms and autistic traits as measured by the Autism-Spectrum Quotient in a non-clinical Japanese population

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Abstract

Introduction: Oxytocin is known to facilitate human behavior and social cognition related to autism. Subclinical autistic traits are continuously distributed in the general population. The aim of this study was to explore the association between oxytocin receptor gene (*OXTR*) polymorphisms and autistic traits.

Methods: Participants consisted of 440 healthy Japanese (272 males and 168 females; mean age 40.9 ± 9.7 years). Participants completed the Japanese version of the Autism-Spectrum Quotient (AQ) and donated a whole blood sample. Fifteen *OXTR* single nucleotide polymorphisms (SNPs) were genotyped using TaqMan or by direct sequencing. Single SNP linear regression analysis, permuted 10,000 times, and haplotype linear regression analysis, were conducted for the AQ and its subscale scores.

Results: Three SNPs – rs2268490, rs2301261, and rs1042778 – were excluded from analysis, as the genotype distributions of rs2268490 were not in Hardy-Weinberg equilibrium, and there were less than 10 minor homozygous participants for rs2301261 and rs1042778. This resulted in 12 SNPs being tested. rs62243370, rs62243369, rs2254298, and rs2268491 were associated with *attention switching* subscale in females by the single SNP analysis. However, after performing Bonferroni corrections, statistical significances were eliminated. The *attention switching* subscale was associated with a specific haplotype, comprising rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, and rs2268491 (GGTGTC, corrected *P* = 0.0016) in females.

Discussion: The present study demonstrated a significant association between a specific *OXTR* haplotype and the autistic trait of "strong focus of attention" as measured by the AQ in a non-clinical female Japanese sample.

Introduction

The neuropeptide oxytocin (OXT) is a nine-amino-acid peptide that acts as a hormone and neurotransmitter; it is synthesized in the hypothalamus and released into the bloodstream and the synaptic cleft

(Uvnäs-Moberg, 2003). OXT facilitates several reproductive processes: it has an effect on uterine contraction and lactation in the peripheries. It regulates parental attachment behaviors (Bartels & Zeki, 2004; Prichard *et al.*, 2007) and social interactions (Uvnäs-Moberg, 1998; Kosfeld *et al.*, 2005; Donaldson &

Young, 2008) as the neurotransmitter. The impairment of social interaction is a central symptom in autism.

The oxytocin receptor gene (*OXTR*) comprises 19.2 kilobases (kb) on chromosome 3p25, and has been identified as one of the putative regions linked to autism (McCauley *et al.*, 2005; Lauritsen *et al.*, 2006). Several studies have found evidence for the association of *OXTR* polymorphisms and autism. Wu *et al.* (2005) investigated four single nucleotide polymorphisms (SNPs) in *OXTR* in Han-Chinese autism spectrum disorder (ASD) trios, and found rs53576 and rs2254298 were significantly associated. Jacob *et al.* (2007) investigated the same two SNPs in a Caucasian sample, and found an association of rs2254298. Lerer *et al.* (2008) found associations of rs1042778 and a haplotype including rs2254298 with ASD in a Caucasian sample. Yrigollen *et al.* (2008) found that rs2268493 was associated with ASD in a Caucasian sample. Liu *et al.* (2010) investigated 11 SNPs in a Japanese sample, and found associations of rs2268491, rs2254298 and a haplotype including rs2254298 with ASD.

Autism is a neuropsychiatric developmental disorder characterized by a triad of features: (i) difficulties with social interaction; (ii) difficulties with communication; and (iii) the presence of restricted, repetitive and stereotyped patterns of behavior, interests and activities (American Psychiatric Association, 2000). The population prevalence is known to be approximately 40 per 10,000 for autism, and over 100 per 10,000 for ASD, with the male-to-female ratio reported as 4:1 (Baird *et al.*, 2006). It is a highly heritable disorder (Bailey *et al.*, 1995; Pickles *et al.*, 1995), with heritability estimated to be above 90% (Rutter, 2000), although the exact mode of transmission is not known.

Autism manifests along a spectrum (Wing, 1997). ASD encompasses the milder variants, including Asperger's Disorder and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) (American Academy of Pediatrics, 2001; Johnson *et al.*, 2007). Subclinical autistic traits are continuously distributed in the general population (Baron-Cohen *et al.*, 2001; Constantino & Todd, 2003) as well as in relatives of individuals with autism designated the broader autism phenotype (Bolton *et al.*, 1994; Bailey *et al.*, 1995, 1998; Le Couteur *et al.*, 1996; Piven *et al.*, 1997). The heritability of autistic traits has been demonstrated in the general population (Bishop *et al.*, 2004; Constantino & Todd, 2005; Constantino *et al.*, 2006).

The Autism-Spectrum Quotient (AQ) was developed to evaluate autistic traits (Baron-Cohen *et al.* 2001). It is a self-administered instrument for measuring the degree to which an adult with normal intel-

ligence has certain traits. It consists of 50 statements assessing personal preferences and habits, with a forced-choice format of "definitely agree", "slightly agree", "slightly disagree", and "definitely disagree". Five subscales, comprising 10 questions each, assess five domains associated with the triad of autistic features noted above (Rutter, 1978; Wing & Gould, 1979; American Psychiatric Association, 2000) as well as demonstrated areas of cognitive abnormality in autism. The five domains assessed are *social skill, attention switching, attention to detail, communication,* and *imagination.* AQ scores range from 0 to 50 points with higher scores indicating a greater degree of autistic traits.

OXTR has many polymorphic sites which have been reported to be associated with autism; autistic traits are also known to show heritability, and are observed both in relatives of individuals with autism and in the general population. We therefore postulate that autistic traits may show an association with *OXTR* polymorphisms in the general population. Thus far, one study examined four *OXTR* SNPs, and found no association with autistic traits as measured by the AQ in a Caucasian sample (Chakrabarti *et al.*, 2009). The present study explored associations among a greater number of *OXTR* SNPs and autistic traits in a non-clinical Japanese population. Haplotypes as well as single SNPs were studied.

Methods

Participants

All participants were recruited in 2008 in Kanagawa Prefecture, adjacent to Tokyo, Japan. They comprised 603 genetically unrelated, non-clinical Japanese white-collar workers in a large corporation, representing a high functioning non-clinical adult population. Participants completed the Japanese version of the AQ (Kurita *et al.*, 2003, 2005), and trained clinicians conducted a short structured diagnostic interview, as per the Mini-International Neuropsychiatric Interview (Sheehan *et al.*, 1998), to confirm lifetime diagnoses of affective, anxiety, and psychotic disorders according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (American Psychiatric Association, 2000). Of the 603 participants, 498 donated a whole blood sample for DNA analysis. Of these 498, five participants were excluded from the study due to ethnic differences (four Chinese and one Russian), and 53 were excluded due to current or past DSM-IV diagnoses: 30 had been diagnosed with major

depressive disorder, 17 with bipolar II disorder, one with dysthymic disorder, one with panic disorder without agoraphobia, two with panic disorder with agoraphobia, and two with agoraphobia without history of panic disorder.

After the above exclusions, the participants of this study comprised 440 high-functioning Japanese (272 males and 168 females). The mean age of participants was 40.9 years (SD = 9.7), with an age range of 23–63 years. Mean ages in males and females were 41.6 (SD = 10.2) and 39.9 (SD = 8.8) years, respectively, with an age range for males of 24–63 years, while for females the range was 23–60 years. No significant differences were observed in demographic data, AQ scores, or AQ subscale scores between the 440 participants and the 163 that were excluded, except for lower scores for *attention switching* ($t = -2.75$, d.f. = 601, $P < 0.01$) and *communication* ($t = -2.24$, d.f. = 601, $P < 0.05$) domains in participants compared to those excluded.

The aim of the present study was clearly explained to all participants, and written informed consent was obtained. The study was approved by the Ethics Committee of the Faculty of Medicine at the University of Tokyo, and conformed to the provisions of the Declaration of Helsinki.

Measurement

The Japanese version of the AQ (Kurita *et al.* 2005) was used to measure autistic traits. The reliability and validity of this version has been confirmed: it demonstrated good reliability (Cronbach's $\alpha = 0.80$ for internal consistency, $r = 0.92$ and mean κ [SD, range] = 0.57 [0.14, 0.28–0.87] for test-retest reliability) and construct validity (Kurita *et al.*, 2003). Subscale scores were calculated by scoring each "slightly agree" and "definitely agree" response as 1, and each "slightly disagree" and "definitely disagree" response as 0. The AQ includes 26 reversed questions, in which a "disagree" response was characteristic for autism. For reversed questions, subscale scores were calculated by scoring each "slightly agree" and "definitely agree" response as 0, and each "slightly disagree" and "definitely disagree" response as 1. Total AQ score was calculated by summing the five subscale scores.

Single nucleotide polymorphism selection and genotyping

Genomic DNA was isolated from leukocytes in whole blood using a Wizard genomic DNA purification kit (Promega Corp., Madison, 2005).

Based on genotype data from the International HapMap Project (International HapMap Consortium, 2003), Haploview 4.2 (Barrett *et al.*, 2005) generated four haplotype blocks plotting 25 tag SNPs with minor allele frequency (mAF) > 5% in *OXTR*. To cover all blocks, seven SNPs whose TaqMan PCR primer and probe sets were available from Assays-On-Demand (http://www3.appliedbiosystems.com) were selected from the 25 SNPs. In addition to these seven SNPs, four SNPs (rs1042778, rs2268493, rs2254298, and rs53576) were selected because they had been reported to be associated with ASD in previous studies (Wu *et al.*, 2005; Jacob *et al.*, 2007; Lerer *et al.* 2008; Yrigollen *et al.*, 2008; Liu *et al.*, 2010). Thus, a total of 11 tag SNPs were selected: rs2301261, rs2268495, rs53576, rs2254298, rs2268493, rs2268491, rs918316, rs2268490, rs237887, rs237885, and rs1042778. These were genotyped using the TaqMan genotyping platform in accordance with the manufacturer's protocol.

Direct sequencing was performed in an 800 b 5′-flanking region of rs2254298, as this SNP had been consistently reported to be associated with autism (Wu *et al.*, 2005; Jacob *et al.*, 2007; Lerer *et al.*, 2008; Liu *et al.*, 2010). The primers were as follows: forward 5′-AGCAGAAACTGTGGGTGTCC-3′ and reverse 5′-CTCTCATCCTCCCTGTGTCC-3′. Sequencing was performed from both 5′ and 3′ ends using the ABI PRISM 3730 Genetic Analyzer in accordance with the manufacturer's protocol (http://www3.appliedbio systems.com). Four SNPs were genotyped by direct sequencing: rs11131149, rs62243370, rs62243369, and rs13316193. Therefore, a total of 15 SNPs were genotyped in this study (rs2301261, rs2268495, rs53576, rs11131149, rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, rs2268491, rs918316, rs2268490, rs237887, rs237885, and rs1042778).

Statistical analysis

First, distributions of AQ scores by sex were examined by histogram. Gender differences in mean AQ scores were assessed by *t*-test.

Second, the Hardy-Weinberg equilibrium (HWE) for genotype distributions was assessed by χ^2 test using Haploview 4.2.

Third, for single SNP-based quantitative trait association analysis, linear regression analyses by single markers were performed for AQ scores with PLINK version 1.07 (Purcell *et al.*, 2007). To reduce the number of multiple tests, an additive model for minor alleles was conducted and other models were not con-

ducted. Family-wise (SNP-wise) corrected empirical *P*-values were calculated on the basis of 10,000 permutations (Good, 2000) under control of the family-wise error rate (FWER) for each SNP examined (Hochberg and Tamhane, 1987).

Finally, linkage disequilibrium (LD) and haplotype were analyzed (Lewontin, 1964). Haplotype block analysis was conducted using the Gabriel method (Gabriel *et al.*, 2002). Haploview was used to obtain the LD. PLINK was used to implement haplotype-based quantitative trait association analysis for AQ scores. Haplotype distributions for each participant were inferred probabilistically using the standard Expectation-Maximization algorithm (Dempster *et al.* 1977). Corrected *P*-values were calculated using the Bonferroni method, as neither Haploview nor PLINK are able to perform permutation tests for haplotype-based quantitative trait association analysis.

The coefficient of determination (R^2) was obtained through regression analysis. R^2 expresses the contribution ratio, and represents the effect size in regression analysis (Cohen, 1988; Field, 2005). R^2 was categorized approximately as small ($0.01 \le R^2 < 0.1$), medium ($0.1 \le R^2 < 0.3$), or large ($0.3 \le R^2$). Statistical power was estimated using QUANTO version 1.2.4 (Gauderman, 2002). Statistical analyses of mean values and SD, χ^2-tests and *t*-tests for demographic data were conducted with SPSS 16.0.2J for Windows (SPSS, Chicago, 2007). Statistical tests were two-tailed and the significance level was set at $P < 0.05$.

Results

Figure 1 shows histograms of total AQ scores by sex. Total AQ scores were continuously distributed in both males and females. Mean scores for the AQ and its five subscales are presented in Table 1. While the difference in the mean total AQ score between males and females did not reach the level of statistical significance, mean scores of males were significantly higher than those of females for the subscales of *attention switching* (t = 2.23, d.f. = 438, *P* < 0.05) and *imagination* (t = 4.90, d.f. = 438, *P* < 0.001).

All 15 SNPs are listed in Table 2. All the SNPs had genotyping call rates of >0.95, and concordance for a duplicate sample was >0.99. The genotype distribution of SNP rs2268490 significantly deviated from HWE (*P* = 0.009), and there were less than 10 minor homozygous participants for rs2301261 and rs1042778. Therefore, these three SNPs were excluded from all subsequent analyses, and the 12 remaining SNPs were tested.
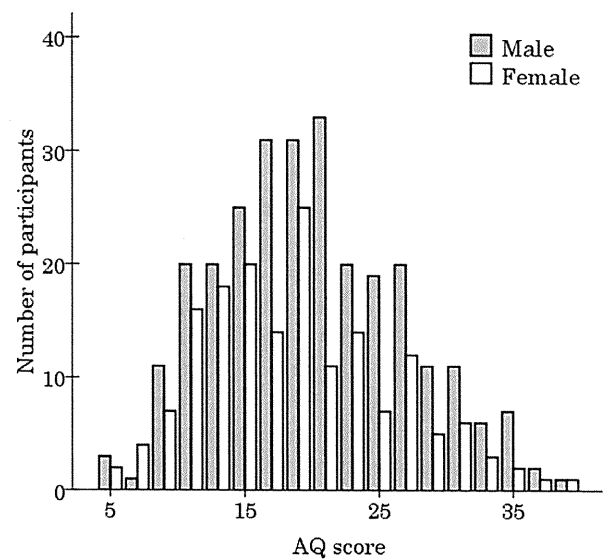
Figure 1 Autism-Spectrum Quotient (AQ) histograms by sex.

Table 1. Mean Autism-Spectrum Quotient (AQ) and subscale scores by sex

Scale/subscale	All (*n* = 440) Mean	SD	Male (*n* = 272) Mean	SD	Female (*n* = 168) Mean	SD
AQ	19.1	7.0	19.6	6.9	18.3	6.9
Social skill	4.5	2.8	4.5	2.8	4.4	2.7
Attention switching	4.1	2.0	4.3	2.0	3.8	2.0
Attention to detail	3.8	2.3	3.7	2.2	4.0	2.3
Communication	2.9	2.2	3.0	3.3	2.8	2.2
Imagination	3.8	1.7	4.1	1.7	3.3	1.7

Based on single SNP linear regression analysis, the *attention switching* subscale showed a SNP-wise empirically significant association with single SNPs (Table 3). However, after making Bonferroni corrections for each subscale and gender subgroup, none of these SNPs exhibited significant associations. None of the AQ scale, other subscales, or other SNPs showed SNP-wise empirically significant associations (Supporting Table S1).

Figure 2 shows Pairwise LD results for the 12 nonexcluded SNPs. A two-haplotype-block structure was found. Block 1 was 2 kb-long and consisted of six SNPs: rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, and rs2268491. Block 2 was 1 kb-long and consisted of two SNPs: rs237887 and rs237885. Block 1 included four specific haplotypes, and block 2 included three, with frequencies > 0.01 (Table 4). Total frequencies of the four haplotypes in block 1, and three haplotypes in block 2, were estimated to be 100.0% and 98.5%, respectively. Based on haplotype linear regression analyses with Bonfer-

Table 2. *OXTR* SNP genotype frequency

SNP	Position	mAF	Genotype	Frequency	n	Total	P-HWE
rs2301261	8785896	0.082	GG / GA / AA	0.843 / 0.150 / 0.007	370 / 66 / 3	439	1.00
rs2268495	8782535	0.236	GG / GA / AA	0.576 / 0.376 / 0.048	253 / 165 / 21	439	0.46
rs53576	8779371	0.366	AA / AG / GG	0.395 / 0.477 / 0.128	173 / 209 / 56	438	0.65
rs11131149	8777852	0.169	GG / GA / AA	0.704 / 0.253 / 0.043	295 / 106 / 18	419	0.06
rs62243370	8777812	0.264	GG / GA / AA	0.534 / 0.404 / 0.062	226 / 171 / 26	423	0.49
rs62243369	8777807	0.266	GG / GA / AA	0.530 / 0.409 / 0.061	224 / 173 / 26	423	0.41
rs13316193	8777745	0.169	TT / TC / CC	0.704 / 0.253 / 0.043	295 / 106 / 18	419	0.06
rs2254298	8777228	0.261	GG / GA / AA	0.536 / 0.405 / 0.059	236 / 178 / 26	440	0.39
rs2268493	8775840	0.156	TT / TC / CC	0.722 / 0.244 / 0.034	317 / 107 / 15	439	0.17
rs2268491	8775398	0.261	CC / CT / TT	0.540 / 0.399 / 0.061	237 / 175 / 27	439	0.58
rs918316	8773181	0.221	TT / TC / CC	0.606 / 0.346 / 0.048	264 / 151 / 21	436	1.00
rs2268490	8772085	0.407	CC / CT / TT	0.320 / 0.546 / 0.134	139 / 237 / 58	434	0.009
rs237887	8772042	0.440	GG / GA / AA	0.312 / 0.497 / 0.191	137 / 218 / 84	439	0.97
rs237885	8770543	0.290	TT / TG / GG	0.506 / 0.409 / 0.085	220 / 178 / 37	435	0.98
rs1042778	8769545	0.102	GG / GT / TT	0.805 / 0.186 / 0.009	350 / 81 / 4	435	1.00

mAF, minor allelic frequency; *OXTR*, oxytocin receptor gene; P-HWE, P-value of Hardy-Weinberg equilibrium; SNP, single nucleotide polymorphism.

Table 3. Results of single SNP linear regression analysis for *attention switching*

	All			Male			Female		
SNP	n	β	P†	n	β	P†	n	β	P†
rs62243370	423	−0.46	0.030	262	−0.15	−	161	−0.92	0.004
rs62243369	423	−0.43	0.047	262	−0.14	−	161	−0.90	0.005
rs2254298	440	−0.41	0.061	272	−0.12	−	168	−0.80	0.014
rs2268491	439	−0.42	0.046	272	−0.16	−	167	−0.77	0.018

†Family-wise (SNP-wise) corrected empirical P-value on the basis of 10,000 permutations. P-values < 0.1 are indicated.
None of the Autism-Spectrum Quotient (AQ) scale, its other subscales, or other SNPs showed SNP-wise empirically significant associations. After using the Bonferroni method to perform multiplicity corrections for each subscale and gender subgroup, none of the SNPs exhibited significant associations.
β, regression coefficient; SNP, single nucleotide polymorphism.

roni multiplicity corrections for each haplotype, block, subscale, and gender subgroup, including each individual SNP analysis, *attention switching* showed a significant association with a haplotype GGTGTC (frequency = 54.9%), in block 1 in females (corrected $P = 0.0016$, $R^2 = 0.12$) (Table 4). The statistical power of the haplotype was estimated at 0.82 for $n = 168$ females, frequency = 0.549, mean *attention switching* score (SD) = 3.83 (2.02), and two-tailed nominal $P < 0.000146$ (=0.05/ ([12 SNPs + 7 haplotypes] × [1 scale + 5 subscales] × [1 group + 2 subgroups]). Haplotype analysis did not show Bonferroni-corrected significant associations for the AQ or other subscales (Supporting Table S2). No significant associations were observed for any specific haplotypes in block 2.

Discussion

OXTR SNPs, including rs62243370, rs62243369, rs2254298, and rs2268491, which exhibited high
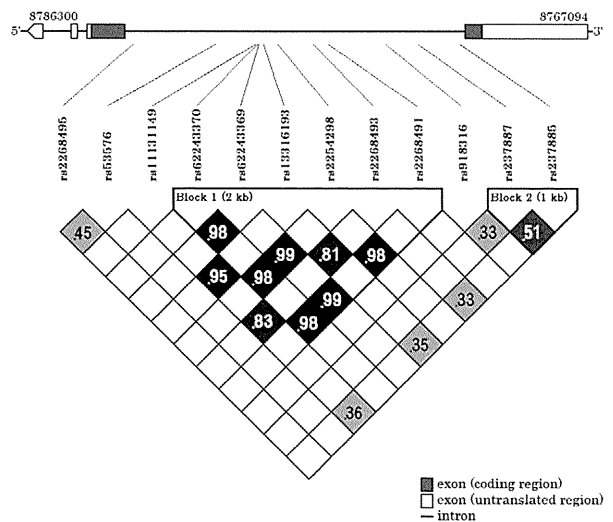
Figure 2 Linkage disequilibrium (LD) plot of the 12 single nucleotide polymorphisms (SNPs). Inter-SNP r^2 of >0.30 is displayed for each pair. D' is >0.8 for all pairs of SNPs in haplotype blocks.

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Table 4. Results of haplotype linear regression analysis for *attention switching*

Block†	Haplotype	All				Male				Female			
		Freq	β	Nom-*P*	Cor-*P*	Freq	β	Nom-*P*	Cor-*P*	Freq	β	Nom-*P*	Cor-*P*
1	GGTGTC	0.568	0.43	0.002	–	0.580	0.05	–	–	0.549	0.95	4.5×10^{-6}	0.0016**
	AATATT	0.252	−0.46	0.005	–	0.246	−0.20	–	–	0.261	−0.89	0.001	–
	GGCGCC	0.150	−0.30	–	–	0.139	0.01	–	–	0.167	−0.71	0.02	–
	GGCGTC	0.015	1.09	0.08	–	0.019	1.16	0.09	–	0.000	NA	NA	NA
2	GT	0.559	0.04	–	–	0.548	−0.11	–	–	0.577	0.35	–	–
	AG	0.290	−0.05	–	–	0.292	0.11	–	–	0.288	−0.30	–	–
	AT	0.151	−0.01	–	–	0.161	0.03	–	–	0.135	−0.17	–	–

**Corrected $P < 0.01$.

†Block 1, rs62243370/rs62243369/rs13316193/rs2254298/rs2268493/rs2268491; Block 2, rs237887/rs237885.

P-values < 0.1 are indicated. None of the Autism-Spectrum Quotient (AQ) or its other subscales showed significant associations after Bonferroni corrections.

β, regression coefficient; Cor-*P*, Bonferroni-corrected *P*-value for multiplicity, for each haplotype, block, subscale, and gender subgroup, including each single SNP analysis; NA, not applicable; Nom-*P*, nominal *P*-value.

inter-SNP r^2 to each other in haplotype block 1 (Figure 2), were associated with *attention switching* in females by the single SNP analysis (Table 3). These findings implied that participants with more major alleles of these SNPs tend to show higher *attention switching*. The single SNP analysis demonstrated an association between the AQ subscale and rs2254298 which was reported to be associated with ASD in previous studies (Wu *et al.*, 2005; Jacob *et al.*, 2007; Lerer *et al.* 2008; Liu *et al.*, 2010). However, after performing Bonferroni corrections, statistical significance was eliminated for all four SNPs.

The six-SNP haplotype block 1, consisting of the four SNPs noted immediately above and two others, showed a Bonferroni-corrected significant association with *attention switching* (Table 4), with medium effect size and power > 0.8. Females with haplotype GGTGTC in block 1 tended to show higher *attention switching*. This haplotype block included rs2254298, which was reported to be associated with ASD.

So far, one study reported no association between *OXTR* SNPs (rs2228485, rs237902, rs237898, and rs237885) and AQ score in a Caucasian sample (Chakrabarti *et al.* 2009). With regard to rs237885, the present study replicated this result, with the Japanese sample showing no association. The reason that their study did not find any associations might be attributable to differences in ethnicity, methods of analysis and/or SNPs. They did not analyze haplotypes, did not differentiate between sex, and did not investigate AQ subscales. Their SNPs did not overlap with our SNPs, except for rs237885. Nor did they examine any SNPs in block 1 which showed a significance in the present study.

Our results indicate that association may differ according to sex (Carter, 2007). The reason for this

difference is not clear. Uhl-Bronner *et al.* (2005) reported gender differences in OXT-binding site expression in the forebrains and spinal cords of rats. Similar differences may exist in the human brain, and may contribute to the regulatory central actions of OXT in human behavior and cognition. This could be related with the present result in gender difference.

This study provided evidence that *OXTR* polymorphism is associated with poor attention switching (or strong focus of attention), which is included in the category of restricted and repetitive interests and behaviors in the autism-specific triad. It has been reported that OXT administration in patients with ASD has led to a reduction in repetitive behaviors (Hollander *et al.*, 2003) and improved social cognition (Hollander *et al.*, 2007). The OXT mechanism in the brain may have a substantial influence on the autism features of restricted and repetitive activities and behavior, as well as difficulty in social interaction.

Limitations to this study include the fact that participants were employees of a major corporation, and were therefore not necessarily representative of the general community: our study participants likely represent a high-functioning segment of the population, and AQ score distribution could be different in the general population. Another limitation is that some SNPs had less than 10 minor homozygous participants, and were therefore excluded from analysis. Due to the small sample size, all selected SNPs could not be examined. A larger sample is needed to detect effects of more SNPs.

In conclusion, the present study demonstrated that one specific *OXTR* haplotype, consisting of rs62243370, rs62243369, rs13316193, rs2254298, rs2268493, and rs2268491, was associated with the

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autistic trait of strong focus of attention with medium effect size and power = 0.82 in a non-clinical Japanese female population.

Supporting information cited in this article is available online.

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