Two genetic variants of CD38 in subjects with autism spectrum disorder and controls

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Two genetic variants of *CD38* in subjects with autism spectrum disorder and controls

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ABSTRACT

The neurobiological basis of autism spectrum disorder (ASD) remains poorly understood. Given the role of CD38 in social recognition through oxytocin (OT) release, we hypothesized that CD38 may play a role in the etiology of ASD. Here, we first examined the immunohistochemical expression of CD38 in the hypothalamus of post-mortem brains of non-ASD subjects and found that CD38 was colocalized with OT in secretory neurons. In studies of the association between *CD38* and autism, we analyzed 10 single nucleotide polymorphisms (SNPs) and mutations of *CD38* by re-sequencing DNAs mainly from a case-control study in Japan, and Caucasian cases mainly recruited to the Autism Genetic Resource Exchange (AGRE). The SNPs of *CD38*, rs6449197 (p<0.040) and rs3796863 (p<0.005) showed significant associations with a subset of ASD (IQ>70; designated as high-functioning autism (HFA)) in the U.S. 104 AGRE family trios, but not with Japanese 188 HFA subjects. A mutation that caused tryptophan to replace arginine at amino acid residue 140 (R140W; (rs1800561, 4693C>T)) was found in 0.6%-4.6% of the Japanese population and was associated with ASD in the smaller case-control study. The SNP was clustered in pedigrees in which the fathers and brothers of T-allele-carrier probands had ASD or ASD traits. In this cohort OT plasma levels were lower in subjects with the T allele than in those without. One proband with the T allele who was taking nasal OT spray showed relief of symptoms. The two variant *CD38* poloymorphysms tested may be of interest with regard of the pathophysiology of ASD.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disease manifesting in childhood but extending through to adulthood. The disorder is more common than previously supposed, with a worldwide frequency of >0.6% (Honda et al., 2005; Baird et al., 2006; Williams et al., 2006). The region with the maximum reported rate (3% of births) is the Nagoya/Hamamatsu region in Japan (Sumi et al., 2006). ASD can be sporadic or familial and is far more common in males than in females (Zhao, 2007). Because ASD is etiologically heterogeneous and forms a continuum, it is likely to involve many genes (Sutcliffe, 2008; Levitt and Campbell, 2009). *De novo* mutations and copy number variations (CNVs) are reported in a small fraction of ASD cases (Sebat et al., 2007; Glesser et al., 2009), but common variants also underlie the disease (Wang et al., 2009), and a unified mechanism for both forms of genetic inheritance has been proposed (Zhao et al., 2007).

Oxytocin (OT) is secreted into the brain by hypothalamic neuronal dendrites and plays important roles in social recognition and memory (Insel & Fernald, 2004; Takayanagi et al., 2005; Donaldson et al., 2008; Neumann, 2008). This hormone mediates behavioral effects, such as pair bonding, mate guarding, and parental care in rodents (Ferguson et al., 2000; Ludwig and Leng, 2006; Campbell, 2008) and may be involved in romantic love, trust, and fear in humans (Koshfeld et al., 2005; Zeki, 2007; Domes et al., 2007; Ditzen et al., 2009). Recently, evidence has accumulating to suggest that the polymorphisms of

multiple OT-related genes are associated with ASD (Wu et al., 2005; Jacob et al., 2007; Ebstein et al. 2008; Gregory et al., 2009; Wermter et al., 2009).

Peripheral or nasal administration of OT facilitates social recognition and trust in healthy humans (Guastella et al., 2008a and b, 2009; Ditzen et al., 2009) and increases eye contact and recognition in autistic subjects (Hollander et al., 2007; Yamasue et al., 2009). Those observations are based mostly on a small number of administrations, and the effects of long-term OT treatment on human social behavior in ASD patients is unknown.

Human CD38 is a type II transmembrane antigen (Malavasi et al., 2008). The *CD38* gene consists of 8 exons on 4p15 (Nakagawa et al., 1995) and spans a genomic stretch of 70.51 kb. The mRNA contains 1227 bases, and single nucleotide polymorphisms (SNPs) have been reported (Nata et al., 1997; Yagui et al., 1998; Ebstein et al., 2009; see Fig. 2A). CD38 has been studied extensively because it is a reliable negative prognostic marker for chronic lymphocytic leukemia (Deaglio et al., 2008). CD38 is expressed in the brain (Lee, 2001; Higashida et al., 2007) and can catalyze the formation of cyclic ADP-ribose (cADPR) from NAD^+ (Lee, 2001; Guse, 2005; Malvasi et al., 2008). cADPR mobilizes Ca^{2+} from intracellular Ca^{2+} stores, thus acting as a second messenger (Lee, 2001). Little was known about the CD38-dependent cADPR/Ca^{2+} signaling pathway in the brain until recent studies in our laboratory. They showed that CD38 regulates OT secretion in the mouse hypothalamus and posterior pituitary, which is critical for mouse social behavior (Jin et al., 2007; Liu et al., 2008). The precise role of CD38 in the human

hypothalamus, however, has not been clarified.

As OT seems to be an important factor for the understanding of ASD, we examined the relationships among human *CD38* polymorphisms and mutations in Japanese, Korean, and Caucasian subjects. We identified two functional polymorphisms in subgroups: ASD and high-functioning autism (HFA) based on IQ (>70 classified as HFA). We measured the carrier's serum OT levels and examined each subject for ASD. Here, we also discuss the possibility of treating ASD patients who have a SNP that lowers OT levels by intranasal administration of OT.

2. **Materials and Methods**

2.1. CD38 expression and immunohistochemistry

We measured CD38 mRNA levels by the semi-quantitative or real-time quantitative RT-PCR method (Jin et al., 2007) using commercially available total RNAs from various regions of the human brain. Control human brain tissues were obtained from archival blocks in the Departments of Pathology at the University of California San Francisco and Kanazawa University Graduate School of Medicine. The use of this tissue followed the institutional guidelines established by the Committee on Human Research (CHR) in both universities. The immunofluorescent stainings for human CD38 and OT were performed according to the procedures described previously (Zhang et al., 2007). Briefly, sections of the hypothalamus were treated with antigen retrieval protocol (0.01M citrate acid buffer pH 6.0, plus heating for 121°C for 5 min). The sections were then incubated with primary

antibody against human CD38 (1:50, sc-7325, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and OT (1:500, AB911, Chemicon, Billerica, MA, USA) overnight in room temperature, followed by washing in Tris-buffered saline (TBS), and incubation with secondary antibodies that were conjugated with Alexa 488 or Alexa 568 (1:300, A11001 and A11011; Invitrogen) for 1 h in room temperature. Images for the co-localization of CD38 and OT in the paraventricular nucleus of the hypothalamus were captured using a Leica confocal microscope (TCS SP, Bannockburn, IL, USA) and imported into the Photoshop software.

2.-2. Participants

The participants consisted of cohorts organized by the Osaka-Hamamatsu-Kanazawa University Joint Research Centers for Child Mental Development, the Kanazawa University COE Program, the Core Research for Evolutional Science and Technology Program in Japan, which includes DNA samples collected at the University of Tokyo (Table 1). Three hundred and fifty-seven ASD subjects were recruited from outpatient psychiatry or pediatric clinics of each university hospital. All subjects fullfilled the DSM-IV criteria for autistic disorder. The diagnoses were made by two experienced child psychiatrists through interviews and clinical record reviews, and the subjects had no apparent physical anomalies. We also recruited patients' parents, grandparents, siblings, and other relatives from 322 families. The controls consisted of unrelated healthy Japanese volunteers—315 from the first cohort and 417 from the second. We recruited adult controls mainly from among hospital and facility staff and medical

schools, and age-matched children as controls. In the Japanese cohorts, all subjects resided in Kanazawa, Hamamatsu/Nagoya, Tokyo, or Osaka, Japan, and all patients and controls were Japanese with no non-Japanese parents or grandparents. Two experienced child psychiatrists independently confirmed the diagnosis of ASD for most patients by semi-structured behavior observation and interviews with the subjects and their parents. At the interview with the parents, which was helpful in the evaluation of autism-specific behaviors and symptoms, the examiner used one of the following instruments: the Asperger Syndrome Diagnostic Interview (Gillberg et al., 2001), Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994), Pervasive Developmental Disorders Autism Society Japan Rating Scale (2006), or Diagnostic Interview for Social and Communication Disorders (Wing et al., 2002).

In addition, for a third case-control study, we recruited 16 male ASD patients and 150 non-ASD male controls from Jeonju University Hospital in Korea. For a fourth study, we recruited 252 families from the Autism Genetic Resource Exchange (http://www.agre.org; AGRE cohort; Geschwind et al., 2001). Additional selection criteria required that (i) there be no possible non-idiopathic autism flags and (ii) all the trios be Whites, with the exclusion of Hispanic and Latino races (Anita et al., 2008). Seven Russian male patients from Krasnoyarsk State Medical University Hospital, and 4 lymphoblastoid cells from Italian male ASD patients from Trino University Medical Hospital were also included in the study. These subjects met the DSM-IV or ADI-R criteria for autistic disorder.

For the HFA group, the U.S. autistic offspring of 104 trios (patient plus two

parents) among the 252 AGRE trios, who had IQ>70, were considered. In the 2nd cohort, we selected 188 trio families as Japanese HFA cases (Table 1).

Using the Autism-Spectrum Quotient (AQ) (Baron-Cohen et al., 2006; Munesue et al., 2008), we evaluated members of families in which older subjects performed self-evaluation by recalling how they behaved in their 20s. Subjects of autism traits in 3 kindreds was defined by AQ scores, above the average (>27) but less than the higher level (<32), during interviews by two psychiatrists.

This study was approved by the ethics committees of Kanazawa University, Hamamatsu Medical University, University of Tokyo, Osaka University, RIKEN, and the other participating institutes.

2.3. Marker selection

The genomic structure of *CD38* is based on the University of California, Santa Cruz, March 2006 draft assembly of the human genome (http://www.genome.ucsc.edu). We selected SNPs using information from the International HapMap Project (http://www.hapmap.org) and the National Center for Biotechnology Information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP). Initially, all the SNPs with MAF>0.1 were selected. Tags, which could capture the common allelic variants with r^2>0.8 by pair-wise tagging, were picked from this set using Haploview v4.0 (http://www.broad.mit.edu/mpg/haploview).

2.4. Genetic analysis

We isolated genomic DNA from venous blood samples using the standard phenol/chloroform method (Easy-DNA kit; Invitrogen, Tokyo, Japan). We amplified *CD38* exons (Tables 2 and 3) and flanking introns (Table 3) by PCR (Taq PCR Core Kit; Qiagen, Hilden, Germany). We used Assay-on-Demand SNP genotyping products (Applied Biosystems, Foster City, CA) to score SNPs based on the TaqMan assay method described previously (Anitha et al., 2008). An ABI 7900 Sequence Detection System (SDS) was used to determine genotypes and analyses were performed with SDS v2.0 software (Applied Biosystems). Fig. 2A shows the SNPs and mutations analyzed in this study and their locations.

2.5. Enzyme immunoassay for OT and vasopressin

Blood samples for measuring OT and vasopressin concentrations were collected in two hospitals in the Kanazawa area between 10:00 and 12:00 or 15:00 and18:00 o'clock from subjects who had been asked to fast for the previous 2 h. Qualified lab technicians drew 10 ml of blood from an arm vein into heparinized tubes in less than 15 min. The samples were centrifuged at 0°C at 2,600 x *g* for 15 min and the plasma was separated off, divided into 2 tubes, and stored at -80°C. We performed the peptide assay for OT and vasopressin (AVP) as described previously (Jin et al., 2007; Liu et al., 2008).

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* 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.

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 1st 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.

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).

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 administration 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 20 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 et al., 2009; Wermter et al., 2009).

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 20 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; Dome et al., 2007; Guastella et al., 2008a and b, 2009). 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 result 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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References

Autism Society Japan, 2006. Pervasive Developmental Disorders Autism Society Japan Rating Scale (PARS). Tokyo, Autism Society Japan.

Anitha, A., Nakamura, K., Yamada, K., Iwayama, Y., Toyota, T., Takei, N., Iwata, Y., Suzuki, K., Sekine, Y., Matsuzaki, H., Kawai, M., Miyoshi, K., Katayama, T., Matsuzaki, S., Baba, K., Honda, A., Hattori, T., Shimizu, S., Kumamoto, N., Tohyama, M., Yoshikawa, T., Mori, N., 2008. Gene and expression analyses reveal enhanced expression of pericentrin 2 (PCNT2) in bipolar disorder. Biol. Psychiatry 63, 678-685.

Arking, D.E., Cutler, D.J., Brune, C.W., Teslovich, T.M., West, K., Ikeda, M., Rea, A., Guy, M., Lin, S., Cook, E.H., Chakravarti, A., 2008. A common genetic variant in the neurexin superfamily member CNTNAP2 increases familial risk of autism. Am. J. Hum. Genet. 82, 160-164.

Baird. G., Simonoff, E., Pickles, A., Chandler, S., Loucas, T., Meldrum, D., Charman, T., 2006. Prevalence of disorders of the autism spectrum in a population cohort of children in South Thames: the Special Needs and Autism Project (SNAP). Lancet 368, 210-215.

Baron-Cohen, S., Hoekstra, R.A., Knickmeyer, R., Wheelwright, S., 2006. The Autism-Spectrum Quotient (AQ)-adolescent version. J. Autism. Dev. Disord. 36, 343-350.
Campbell, A., 2008. Attachment, aggression and affiliation: the role of oxytocin in female social behavior. Biol. Psychol. 77, 1-10.

Deaglio, S., Aydin, S., Vaisitti, T., Bergui, L., Malavasi, F., 2008. CD38 at the junction between prognostic marker and therapeutic target. Trends Mol. Med. 14, 210-218.

Ditzen, B., Schaer, M., Gabriel, B., Bodenmann, G., Ehlert, U., Heinrichs, M. 2009. Intranasal oxytocin increases positive communication and reduces cortisol levels during couple conflict. Biol. Psychiatry 65, 728-731.

Domes, G., Heinrichs, M., Michel, A., Berger, C., Herpertz, S.C., 2007. Oxytocin improves "mind-reading" in humans. Biol. Psychiatry 61, 731-733.

Donaldson, Z.R., Young, L.J., 2008. Oxytocin, vasopressin, and the neurogenetics of sociality. Science 322, 900-904.

Ebstein, R.P., Israel, S., Lerer, E., Uzefovsky, F., Shalev, I., Gritsenko, I., Riebold, M., Salomon, S., Yirmiya, N., 2009. Arginine vasopressin and oxytocin modulate human social behavior. Ann. N. Y. Acad. Sci.1167, 87-102.

Ferguson, J.N., Young, L.J., Hearn, E.F., Matzuk, M.M., Insel, T.R., Winslow, J.T., 2000. Social amnesia in mice lacking the oxytocin gene. Nature Genet. 25, 284-288.

Geschwind, D.H, Sowinski, J., Lord, C., Iversen, P., Shestack, J.,, Jones, P., Ducat, L., Spence, S.J.; AGRE Steering Committee., 2001. The autism genetic resource exchange: a resource for the study of autism and related neuropsychiatric conditions. Am. J. Hum. Genet. 69, 463-466.

Gillberg, C., Gillberg, C., Rastam, M., Wentz, E., 2001. The Asperger Syndrome (and high-functioning autism) Diagnostic Interview (ASDI): a preliminary study of a new structured clinical interview. Autism 5, 57-66.

Glessner, J.T., Wang, K., Cai, G., Korvatska, O., Kim, C.E., Wood, S., Zhang, H., Estes, A., Brune, C.W., Bradfield, J.P., Imielinski, M., Frackelton, E.C., Reichert, J., Crawford, E.L., Munson, J., Sleiman, P.M., Chiavacci, R., Annaiah, K., Thomas, K., Hou, C., Glaberson, W., Flor, J., Otieno, F., Garris, M., Soorya, L., Klei, L., Piven, J., Meyer, K.J., Anagnostou, E., Sakurai, T., Game, R.M., Rudd, D.S., Zurawiecki, D., McDougle, C.J., Davis, L.K., Miller, J., Posey, D.J., Michaels, S., Kolevzon, A., Silverman, J.M., Bernier, R., Levy, S.E., Schultz, R.T., Dawson, G., Owley, T., McMahon, W.M., Wassink, T.H., Sweeney, J.A., Nurnberger, J.I., Coon, H., Sutcliffe, J.S., Minshew, N.J., Grant, S.F., Bucan, M., Cook, E.H., Buxbaum, J.D., Devlin, B., Schellenberg, G.D., Hakonarson, H., 2009. Autism genome-wide copy number variation reveals ubiquitin and neuronal genes.

Nature 459, 569-573.

Gregory, S.G., Connelly, J.J., Towers, A.J., Johnson, J., Biscocho, D., Markunas, C.A., Lintas, C., Abramson, R.K., Wright, H.H., Ellis, P., Langford, C.F., Worley, G., Delong, G.R., Murphy, S.K., Cuccaro, M.L., Persico, A., Pericak-Vance, M.A., 2009. Genomic and epigenetic evidence for oxytocin receptor deficiency in autism. BMC Med. 7, 62.

Guastella, A.J., Mitchell, P.B., Dadds, M.R., 2008. Oxytocin increases gaze to the eye region of human faces. Biol. Psychiatry 63, 3-5.

Guastella, A.J., Mitchell, P.B., Mathews, F., 2008. Oxytocin enhances the encoding of positive social memories in humans. Biol. Psychiatry 64, 256-258.

Guastella, A.J., Einfeld, S.L., Gray, K.M., Rinehart, N.J., Tonge, B.J., Lambert, T.J., Hickie, I.B., 2009. Intranasal Oxytocin Improves Emotion Recognition for Youth with Autism Spectrum Disorders. Biol. Psychiatry. In press.

Guse, A.H., 2005. Second messenger function and the structure-activity relationship of cyclic adenosine diphosphoribose (cADPR). FEBS J. 272, 4590-4597.

Hayward, S., Lee, R.A., 2002. Improvements in the analysis of domain motions in proteins from conformational change: DynDom version 1.50. J. Mol. Graph. Model 21, 181-183.

Higashida, H., Salmina, A.B., Olovyannikova, R.Y., Hashii, M., Yokoyama, S., Koizumi, K., Jin, D., Liu, H.X., Lopatina, O., Amina, S., Islam, M.S., Huang, J.J., Noda, M., 2007. Cyclic ADP-ribose as a universal calcium signal molecule in the nervous system. Neurochem. Int. 51, 192-199.

Hollander, E., Bartz, J., Chaplin, W., Phillips, A., Sumner, J., Soorya, L., Anagnostou, E., Wasserman, S., 2007. O xytocin increases retention of social cognition in autism. Biol. Psychiatry 61, 498-503.

Honda, H., Shimizu, Y., Imai, M., Nitto, Y., 2005. Cumulative incidence of childhood autism: a total population study of better accuracy and precision. Dev. Med. Child Neurol. 47, 10-18.

Insel, T.R., Fernald, R.D., 2004. How the brain processes social information: searching for the social brain. Annu. Rev. Neurosci. 27, 697-722.

Jacob, S., Brune, C.W., Carter, C.S., Leventhal, B.L., Lord, C., Cook, E.H. Jr., 2007. Association of the oxytocin receptor gene (OXTR) in Caucasian children and adolescents with autism. Neurosci Lett. 417, 6-9.

.

Jamroziak, K., Szemraj, Z., Grzybowska-Izydorczyk, O., Szemraj, J., Bieniasz, M., Cebula, B., Giannopoulos, K., Balcerczak, E., Jesionek-Kupnicka, D., Kowal, M., Kostyra, A., Calbecka, M., Wawrzyniak, E., Mirowski, M., Kordek, R., Robak, T., 2009. CD38 gene polymorphisms contribute to genetic susceptibility to B-cell chronic lymphocytic leukemia: evidence from two case-control studies in Polish Caucasians. Cancer Epidemiol. Biomarkers Prev. 18, 945-953.

Jin, D., Liu, H.X., Hirai, H., Torashima, T., Nagai, T., Lopatina, O., Shnayder, N.A., Yamada, K., Noda, M., Seike, T., Fujita, K., Takasawa, S., Yokoyama, S., Koizumi, K., Shiraishi, Y., Tanaka, S., Hashii, M., Yoshihara, T., Higashida, K., Islam, M.S., Yamada, N., Hayashi, K., Noguchi, N., Kato, I., Okamoto, H., Matsushima, A., Salmina, A., Munesue, T., Shimizu, N., Mochida, S., Asano, M., Higashida, H., 2007. CD38 is critical for social behaviour by regulating oxytocin secretion. Nature 446, 41-45.

Lee, H.C., 2001. Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. Annu. Rev. Pharmacol. Toxicol. 41, 317-345.

Liu, H.X., Lopatina, O., Higashida, C., Tsuji, T., Kato, I., Takasawa, S., Okamoto, H., Yokoyama, S., Higashida, H., 2008. Locomotor activity, ultrasonic vocalization and oxytocin levels in infant CD38 knockout mice. Neurosci. Lett. 448, 67-70.

Ludwig, M., Leng, G., 2006. Dendritic peptide release and peptide-dependent behaviours. Nat. Rev. Neurosci. 7, 126-136.

Kosfeld, M., Heinrichs, M., Zak, P.J., Fischbacher, U., Fehr, E., 2005. Oxytocin increases trust in humans. Nature 435, 673-676.

Levitt, P., Campbell, D.B., 2009. Genetic evidence implicating multiple genes in the MET receptor tyrosine kinase pathway in autism spectrum disorder. J. Clin. Invest. 119, 747-754.

Lord, C., Rutter, M., Le, Couteur, A., 1994. Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. J. Autism Dev. Disord. 24, 659-685.

Mallone, R., Ortolan, E., Baj, G., Funaro, A., Giunti, S., Lillaz, E., Saccucci, F., Cassader, M., Cavallo-Perin, P., Malavasi, F., 2001. Autoantibody response to CD38 in Caucasian patients with type 1 and type 2 diabetes: immunological and genetic characterization. Diabetes 50, 752-762.

Malavasi, F., Deaglio, S., Funaro, A., Ferrero, E., Horenstein, A.L., Ortolan, E., Vaisitti, T., Aydin, S., 2008. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. Physiol. Rev. 88, 841-886.

Munesue, T., Ono, Y., Mutoh, K., Shimoda, K., Nakatani, H., Kikuchi, M., 2008. High prevalence of bipolar disorder comorbidity in adolescents and young adults with high-functioning autism spectrum disorder: a preliminary study of 44 outpatients. J. Affect. Disord. 111, 170-175.

Nakagawara, K., Mori, M., Takasawa, S., Nata, K., Takamura, T., Berlova, A., Tohgo, A., Karasawa, T., Yonekura, H., Takeuchi, T., Okamoto, H. 1995. Assignment of CD38, the gene encoding human leukocyte antigen CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase), to chromosome 4p15. Cytogenet. Cell Genet. 69, 38-39.

Nata, K., Takamura, T., Karasawa, T., Kumagai, T., Hashioka, W., Tohgo, A., Yonekura, H., Takasawa, S., Nakamura, S., Okamoto, H., 1997. Human gene encoding CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase): organization, nucleotide sequence and alternative splicing. Gene 186, 285-92.

Neumann, I.D., 2008. Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. J. Neuroendocrinol. 20, 858-865.

Ranade, K., 2001. High-throughput genotyping with single nucleotide polymorphisms. Genome Res. 11, 1262-1268.

Sebat, J., Lakshmi, B., Malhotra, D., Troge, J., Lese-Martin, C., Walsh, T., Yamrom, B., Yoon, S., Krasnitz, A., Kendall, J., Leotta, A., Pai, D., Zhang, R., Lee, Y.H., Hicks, J., Spence, S.J., Lee, A.T., Puura, K., Lehtimäki, T., Ledbetter, D., Gregersen, P.K., Bregman, J., Sutcliffe, J.S., Jobanputra, V., Chung, W., Warburton, D., King, M.C., Skuse, D., Geschwind, D.H., Gilliam, T.C., Ye, K., Wigler, M., 2007. Strong association of de novo copy number mutations with autism. Science 316, 445-449.

Sumi, S., Taniai, H., Miyachi, T., Tanemura, M., 2006. Sibling risk of pervasive developmental disorder estimated by means of an epidemiologic survey in Nagoya, Japan. J, Hum. Genet. 51, 518-522.

Sutcliffe, J.S., 2008. Heterogeneity and the design of genetic studies in autism. Autism Res. 1, 205-206.

Takayanagi, Y., Yoshida, M., Bielsky, I.F., Ross, H.E., Kawamata, M., Onaka, T., Yanagisawa, T., Kimura, T., Matzuk, M.M., Young, L.J., Nishimori, K., 2005. Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. Proc. Natl. Acad. Sci. USA 102, 16096-16101.

Wang, K., Zhang, H., Ma, D., Bucan, M., Glessner, J.T., Abrahams, B.S., Salyakina, D., Imielinski, M., Bradfield, J.P., Sleiman, P.M., Kim, C.E., Hou, C., Frackelton, E.,

Chiavacci, R., Takahashi, N., Sakurai, T., Rappaport, E., Lajonchere, C.M., Munson, J., Estes, A., Korvatska, O., Piven, J., Sonnenblick, L.I., Alvarez Retuerto, A.I., Herman, E.I., Dong, H., Hutman, T., Sigman, M., Ozonoff, S., Klin, A., Owley, T., Sweeney, J.A., Brune, C.W., Cantor, R.M., Bernier, R., Gilbert, J.R., Cuccaro, M.L., McMahon, W.M., Miller, J., State, M.W., Wassink, T.H., Coon, H., Levy, S.E., Schultz, R.T., Nurnberger, J.I., Haines, J.L., Sutcliffe, J.S., Cook, E.H., Minshew, N.J., Buxbaum, J.D., Dawson, G., Grant, S.F., Geschwind, D.H., Pericak-Vance, M.A., Schellenberg, G.D., Hakonarson, H., 2009. Common genetic variants on 5p14.1 associate with autism spectrum disorders. Nature 459, 528-533.

Weiss, L.A., Arking, D.E.; Gene Discovery Project of Johns Hopkins & the Autism Consortium, Daly, M.J., Chakravarti, A., 2009. A genome-wide linkage and association scan reveals novel loci for autism. Nature 461, 802-808.

Wermter, A.K., Kamp-Becker, I., Hesse, P., Schulte-Körne, G., Strauch, K., Remschmidt, H., 2009. Evidence for the involvement of genetic variation in the oxytocin receptor gene (OXTR) in the etiology of autistic disorders on high-functioning level. Am. J. Med. Genet. B Neuropsychiatr. Genet. In press

Williams, J.G., Higgins, J.P., Brayne, C.E., 2006. Systematic review of prevalence studies of autism spectrum disorders. Arch. Dis. Child 91, 8-15.

Wing, L., Leekam, S.R., Libby, S.J., Gould, J., Larcombe, M., 2002. The Diagnostic Interview for Social and Communication Disorders: background,inter-rater reliability and clinical use. J. Child Psychol. Psychiat. 43, 307-325.

Wu, S., Jia, M., Ruan, Y., Liu, J., Guo, Y., Shuang, M., Gong, X., Zhang, Y., Yang, X., Zhang, D., 2005. D.Positive association of the oxytocin receptor gene (OXTR) with autism in the Chinese Han population. Biol Psychiatry. 58, 74-77.

Yagui, K., Shimada, F., Mimura, M., Hashimoto, N., Suzuki, Y., Tokuyama, Y., Nata, K., Tohgo, A., Ikehata, F., Takasawa, S., Okamoto, H., Makino, H., Saito, Y., Kanatsuka, A.,

1998. A missense mutation in the CD38 gene, a novel factor for insulin secretion: association with Type II diabetes mellitus in Japanese subjects and evidence of abnormal function when expressed in vitro. Diabetologia 41, 1024-1028.

Yamashita, M., Glasgow, E., Zhang, B.J., Kusano, K., Gainer, H., 2002. Identification of cell-specific messenger ribonucleic acids in oxytocinergic and vasopressinergic magnocellular neurons in rat supraoptic nucleus by single-cell differential hybridization. Endocrinology 143, 4464-4476.

Yamasue, H., Kuwabara, H., Kawakubo, Y., Kasai, K., 2009. Oxytocin, sexually dimorphic features of the social brain, and autism. Psychiatry Clin. Neurosci. 63, 129-140.

Zeki, S., 2007. The neurobiology of love. FEBS Lett. 581, 2575-2579.

Zhang, J., Pho, V., Bonasera, S.J., Holtzman, J., Tang, A.T., Hellmuth, J., Tang, S., Janak, P.H., Tecott, L.H., Huang, E.J., 2007. Essential function of HIPK2 in TGFbeta-dependent survival of midbrain dopamine neurons. Nat. Neurosci. 10, 77-86.

Zhao, X., Leotta, A., Kustanovich, V., Lajonchere, C., Geschwind, D.H., Law, K., Law, P., Qiu, S., Lord, C., Sebat, J., Ye, K., Wigler, M., 2007. A unified genetic theory for sporadic and inherited autism. Proc. Natl. Acad. Sci. USA 104, 12831-12836

Figure legends

Fig. 1. Immunohistochemical analysis of CD38 and oxytocin in the human brain. Cell montages of panels were taken from the paraventricular nucleus (PVN) in the hypothalamus (A to C) and insular cortex (D to F) of autopsy subjects from the USA. Arrow heads indicate extensive colabeling with OT. The insets in panels are enlarged images of neurons showing coexpression of CD38 and OT. Scale bars: 40 μm in C and 8 μm in insert.

Fig. 2. Genome structure, SNPs, and mutations of *CD38*. (A) Genomic structure of *CD38* and locations of SNPs in introns (upper) and mutations in 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.

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.

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 numbers. The arrow indicates the proband. *, Autism trait; #, Autism; A, Asperger.

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 (5-9ns). 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.

Table 1

Data sets for the 4 case-control study cohorts.

Data set*	Cohort 1	Cohort 2	Cohort 3	Cohort 4
	Subjects Age (Range) Male/Female	Subjects Age (Range) Male/Female	Subjects	Subjects Male/Female
Cases (Probands)	**29** 22.8 ± 7.6 (12-44) 23/6	**301** 11.9 ± 6.7 (3-64) 263/38.	**16**	**263** 263/0
Controls	**315** 34.1 ± 4.3 (8-75) 171/144	**417** 28.6 ± 14.4 (5-65) 229/188	**150**	-
Trio families	**3**	**334**		**252**
Family member	**25** 53.0 ± 4.5 (21-84) 15/10	**297** 39.2 ± 15.5 (3-93) 143/154	-	-
HFA subjects	-	**188**		**104**
Usage/analysis	Intronic SNP	-	-	Intronic SNP
	Exonic mutation	R140W	R140W	R140W
	Family-based associatio	HFA association		HFS association
	OT/AVP measurement			

*Cohort 1 was from the Kanazawa area, Japan; Cohort 2, the Nagoya/Hamamatsu, Tokyo, and Osaka areas, Japan; Cohort 3, Jeonju, Korea; Cohort 4, study of 252 trio samples from the Autism Genetic Resource Exchange comprising 252 U.S., 7 Russian, and 4 Italian ASD patients. Age ± SEM years, (Year range).

Table 2

Oligonucleotides. Designed for amplification of coding sequences including 60–100 bp of flanking intronic sequences. Primers were followings:

Exon	Up	Down
1	5’-AGGGAAACAGAGAAAAGGCAAGTGU	5’-GGCCAGCTGCTCCTGAAAG-3’
2	5’-GGCATATAATAGATGCTTCC-3’	5’-TGGACCTATGAATTGTTACC-3’
3	5’-GACATGCTAAATTGATCTCAG-3’	5’-CAGCAGAAGTCACTCTGTTC-3’
4	5’-TCCACTATGACTGAACAGCC-3’	5’-AGCACTGACTGAGTAACG-3’
5	5’-CTTAACCAGCTATTGCTAAG	5’-ACTGTGATATTTGCAACAGG-3’
6	5’-TCTGCCTGCTGGTTGTTGAG-3	5’-TCCTGAGTCAATTTGTTCCC-3’
7	5’-CCCAACAGCCTCTTAACTTT-3’	5’-ATCACCAGAGGTTGCCAT-3’
8	5’-AGCGAATTGGACGACAGATG-3’	5’-CATTGACCTTATTGTGGAGG-3’

Usually, we used the following temperatures: 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at annealing temperature at 52 °C, 5 min at 72 °C for extension followed by a final extension of 10 min at 72 °C. SNPs of these samples were examined by the methods described below.

Table 3

Sequence information for SNPs1-10 and R140W.

SNP	Sequence	Strand	ON GENE	UCSC NO
SNP01 (rs3796878)	TTGCTCTGTTCCCAGGTTGGTCCTCC[A/G]CATACCTCCCACTGCAGGATCCTGG	-	T/C	15,394,757
SNP02 (rs3796875)	GTTTTTCAAGAGTCCTAAGACAAAGA[A/G]GAAAGGAAGAAGCAGAGAAGCCATG	-	T/C	15,396,312
SNP03 (rs6449197)	CAGGTTGAGGAAATTTTATTTCTAAT[C/T]TGCTCAGTGTTTTTTTCATCACAAG	+	C/T	15,424,020
SNP04 (rs11574927)	AAAATTGTGTACCCCAATTCAGTAGT[A/G]AAACTACTACCGGGAACATCGGGAA	+	A/G	15,449,341
SNP05 (rs10805347)	ATTAACATTTCAGAATTTTATGATCT[A/G]ATATTATGGTTCAAGCACTTGAAAC	+	A/G	15,449,937
SNP06 (rs3796863)	GGGAGGGGAGCTATCCATGCCACCTG[A/C]TGGTCAAAAAAACAGCAGGAGCAGC	-	T/G	15,459,084
SNP07 (rs1130169)	TGTACCCTTCCT ACAGATAGTCAAAC[C/T]ATAAACTTCATGGTCATGGGTCATG	+	C/T	15,459,783
SNP08 8rs13137313)	AAATAAACCATATGTGTTGAACAAAG[A/G]ATTAATAAATTAATTTGAGACTCAA	+	A/G	15,461.066
SNP09 (RS3733593)	ATCTTGAACAAAATCGCCTAACCTTTC[C/T]GAACTTCAACTTCCTTGCCACTCCT	+	C/T	15,461,202
SNP10 (rs3733593)	CTGCCTCCGAATTCCATAGTTTCCAC[C/T]GCCTTGCGCTACTTGCATTCTCTGATT	-	G/A	15,463,823
R140W (rs1800561)	TGGCCCATCAGTTCACACAGGTCCAG[C/T]GGGACATGTTCACCCTGGAGGACAC	+	C/T	15,435,656

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

Marker	Allele	252 trios				HFA trios			
		Families*	Frequency	Z-score	p-value	Families*	Frequency	Z-score	p-value†
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

* Informative families.

† Significant *p*-values are indicated in bold italic.

Table 5

Haplotype associations of SNPs belonging to the three LD blocks of *CD38* in HFA trio

Block	Haplotype*	Frequency	T (%)	Individual *p*-value	Permutation ** *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). *All possible combinations of haplotypes with frequency >0.01. Significant values ($p<0.05$) are indicated in bold italic. **10,000 permutations.

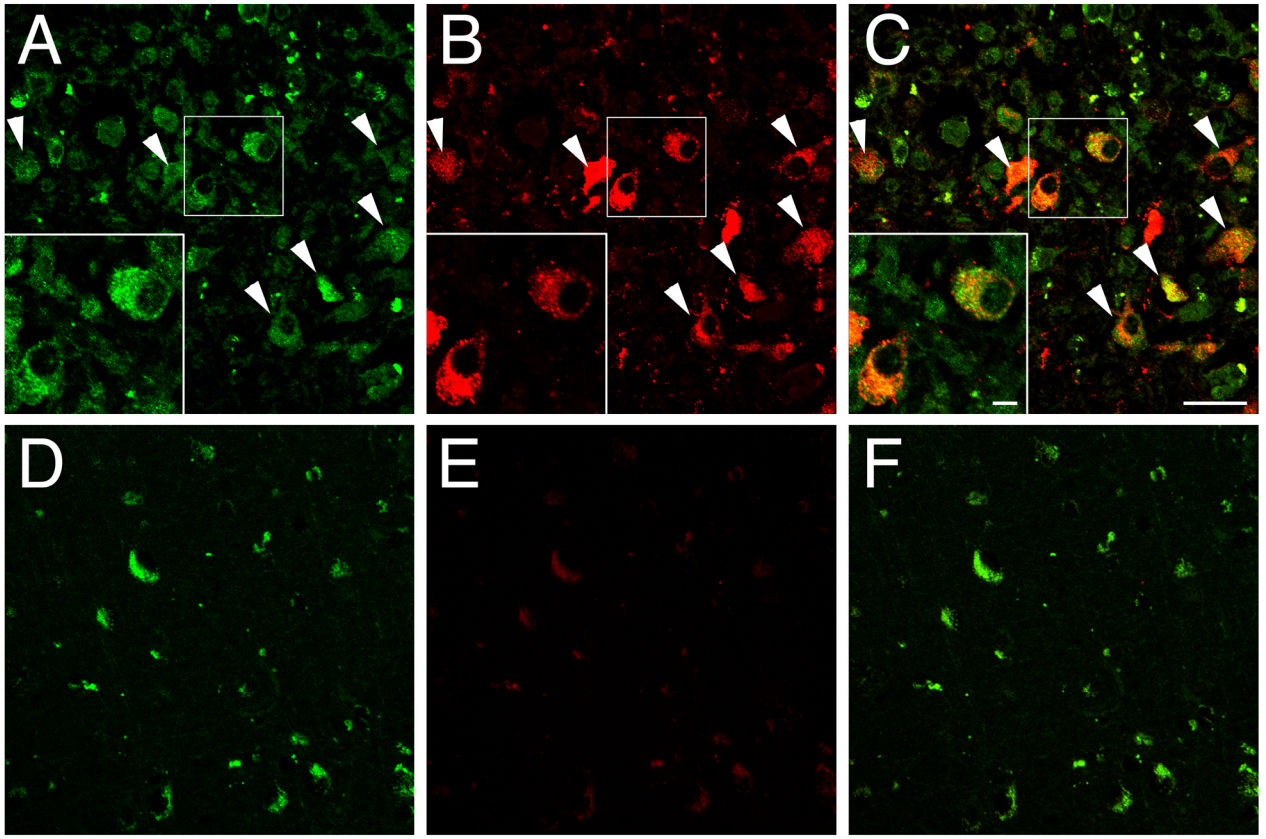


Fig. 1

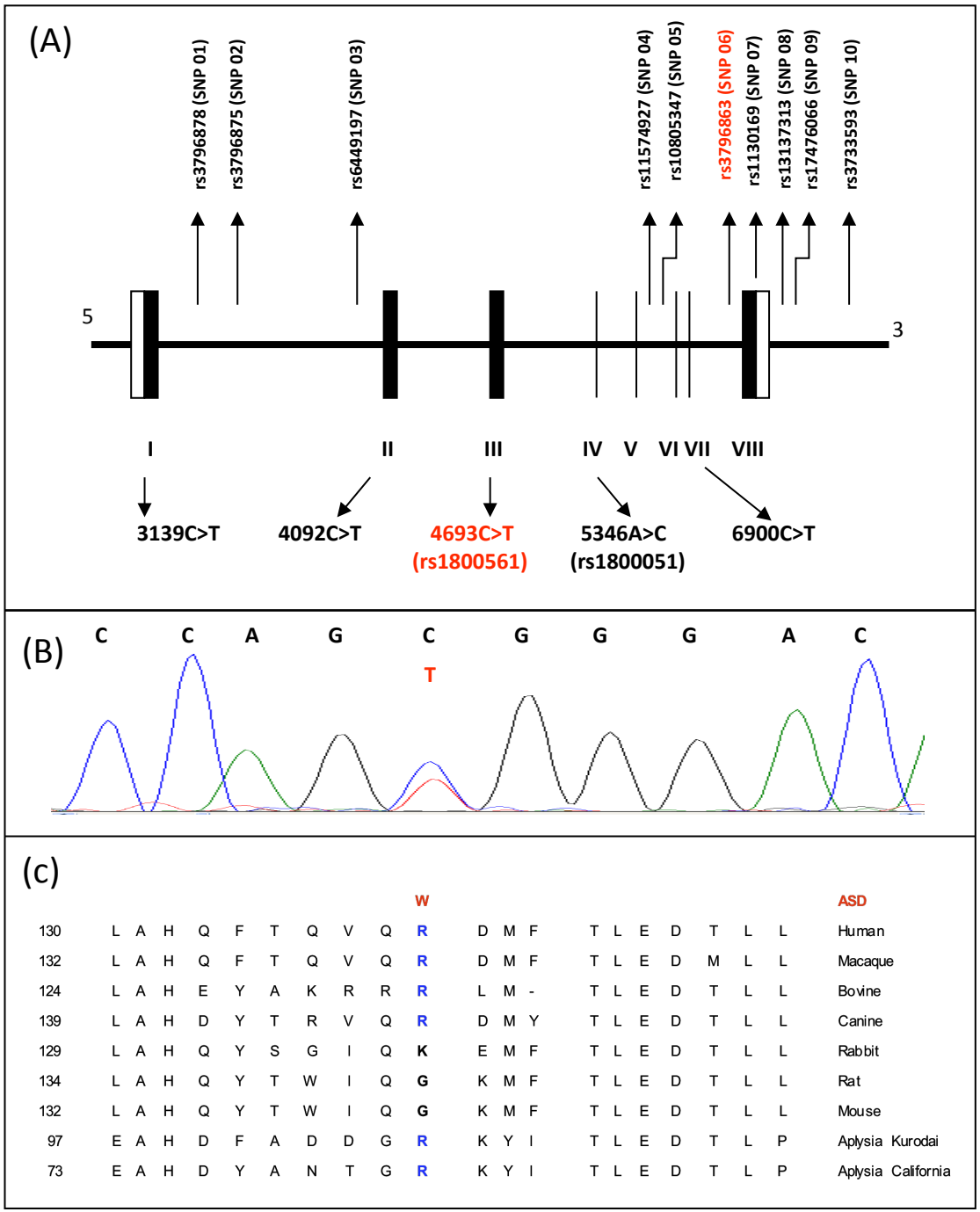
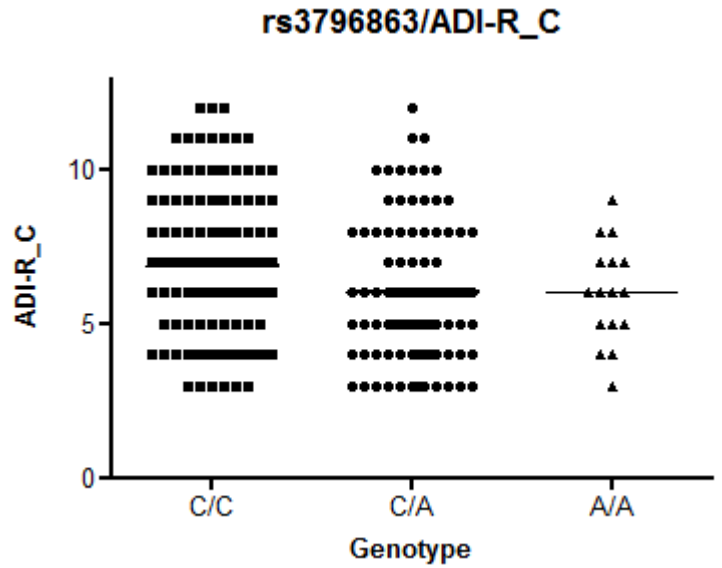

(A)
rs3796878 (SNP 01)
rs3796875 (SNP 02)
rs6449197 (SNP 03)
rs11574927 (SNP 04)
rs10805347 (SNP 05)
rs3796863 (SNP 06)
rs1130169 (SNP 07)
rs13137313 (SNP 08)
rs17476066 (SNP 09)
rs3733593 (SNP 10)
5
3
I
II
III
IV
V
VI
VII
VIII
3139C>T
4092C>T
4693C>T
(rs1800561)
5346A>C
(rs1800051)
6900C>T
(B)
C C A G C G G G A C
T
(c)
W
ASD
130 L A H Q F T Q V Q R D M F T L E D T L L Human
132 L A H Q F T Q V Q R D M F T L E D M L L Macaque
124 L A H E Y A K R R R L M - T L E D T L L Bovine
139 L A H D Y T R V Q R D M Y T L E D T L L Canine
129 L A H Q Y S G I Q K E M F T L E D T L L Rabbit
134 L A H Q Y T W I Q G K M F T L E D T L L Rat
132 L A H Q Y T W I Q G K M F T L E D T L L Mouse
97 E A H D F A D D G R K Y I T L E D T L P Aplysia Kurodai
73 E A H D Y A N T G R K Y I T L E D T L P Aplysia California

Fig. 2

(A)


rs3796863/ADI-R_C
ADI-R_C
10
5
0
C/C
C/A
A/A
Genotype

(B)

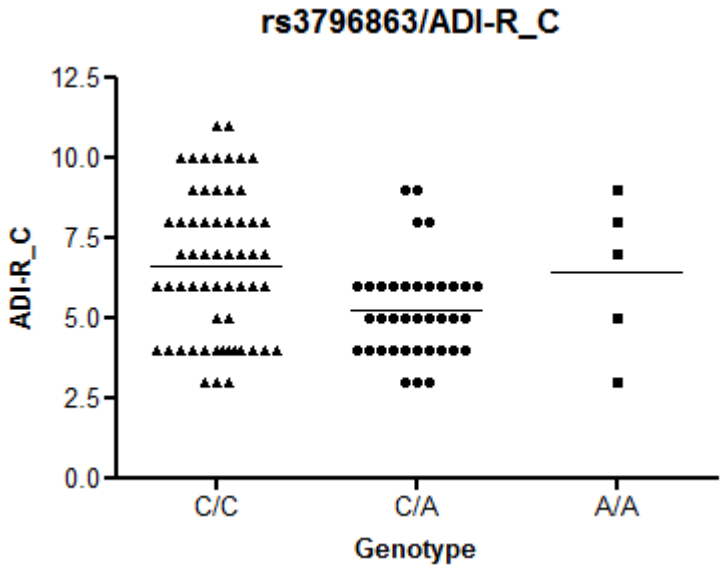

rs3796863/ADI-R_C
ADI-R_C
12.5
10.0
7.5
5.0
2.5
0.0
C/C
C/A
A/A
Genotype

Fig. 3

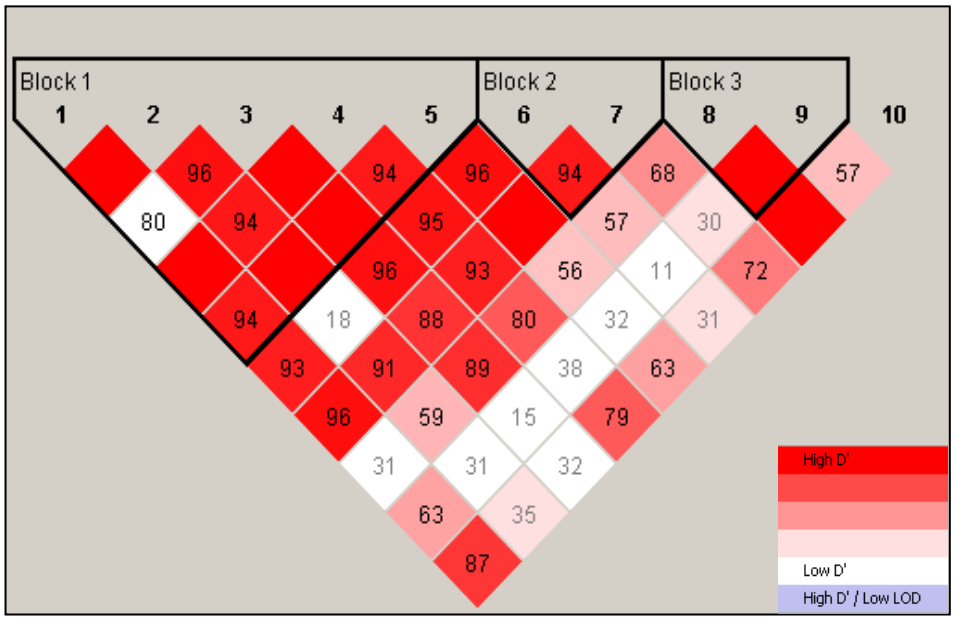

Block 1
Block 2
Block 3
1
2
3
4
5
6
7
8
9
10
96
94
96
94
68
57
80
94
95
57
30
96
93
56
11
72
94
18
88
80
32
31
93
91
89
38
63
96
59
15
79
31
31
32
63
35
87
High D'
Low D'
High D' / Low LOD

Fig. 4

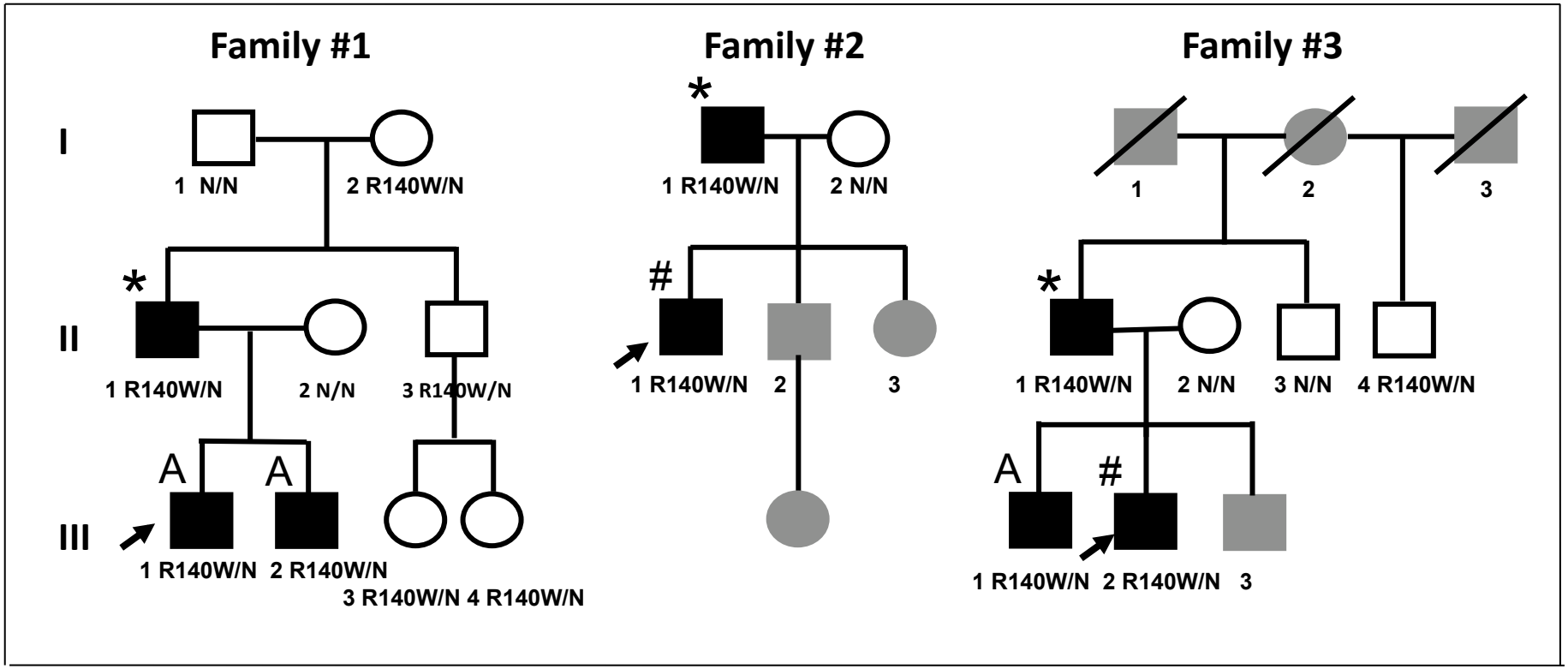

Family #1
I
1 N/N
2 R140W/N
*
II
1 R140W/N
2 N/N
3 R140W/N
A
A
III
1 R140W/N
2 R140W/N
3 R140W/N
4 R140W/N
Family #2
*
1 R140W/N
2 N/N
#
1 R140W/N
2
3
Family #3
1
2
3
*
1 R140W/N
2 N/N
3 N/N
4 R140W/N
A
#
1 R140W/N
2 R140W/N
3

Fig. 5

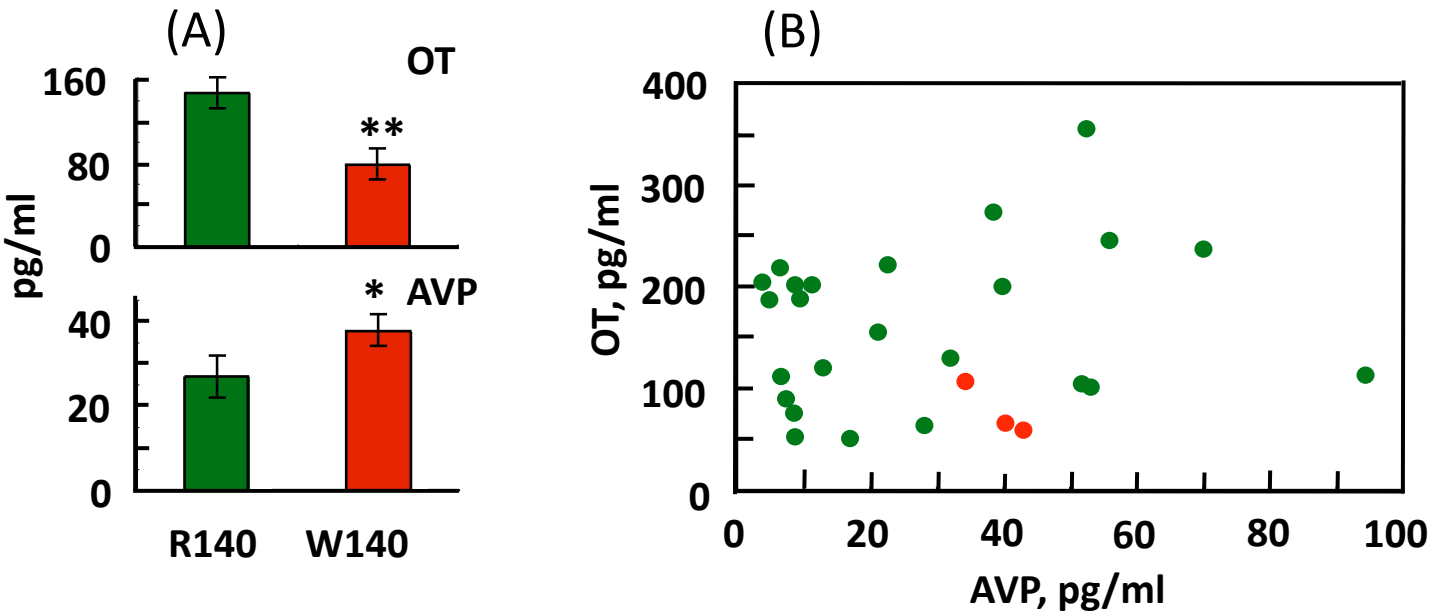

(A)
OT
160
80
0
pg/ml
**
* AVP
40
20
0
R140
W140
(B)
400
300
200
100
0
OT, pg/ml
0
20
40
60
80
100
AVP, pg/ml

Fig. 6

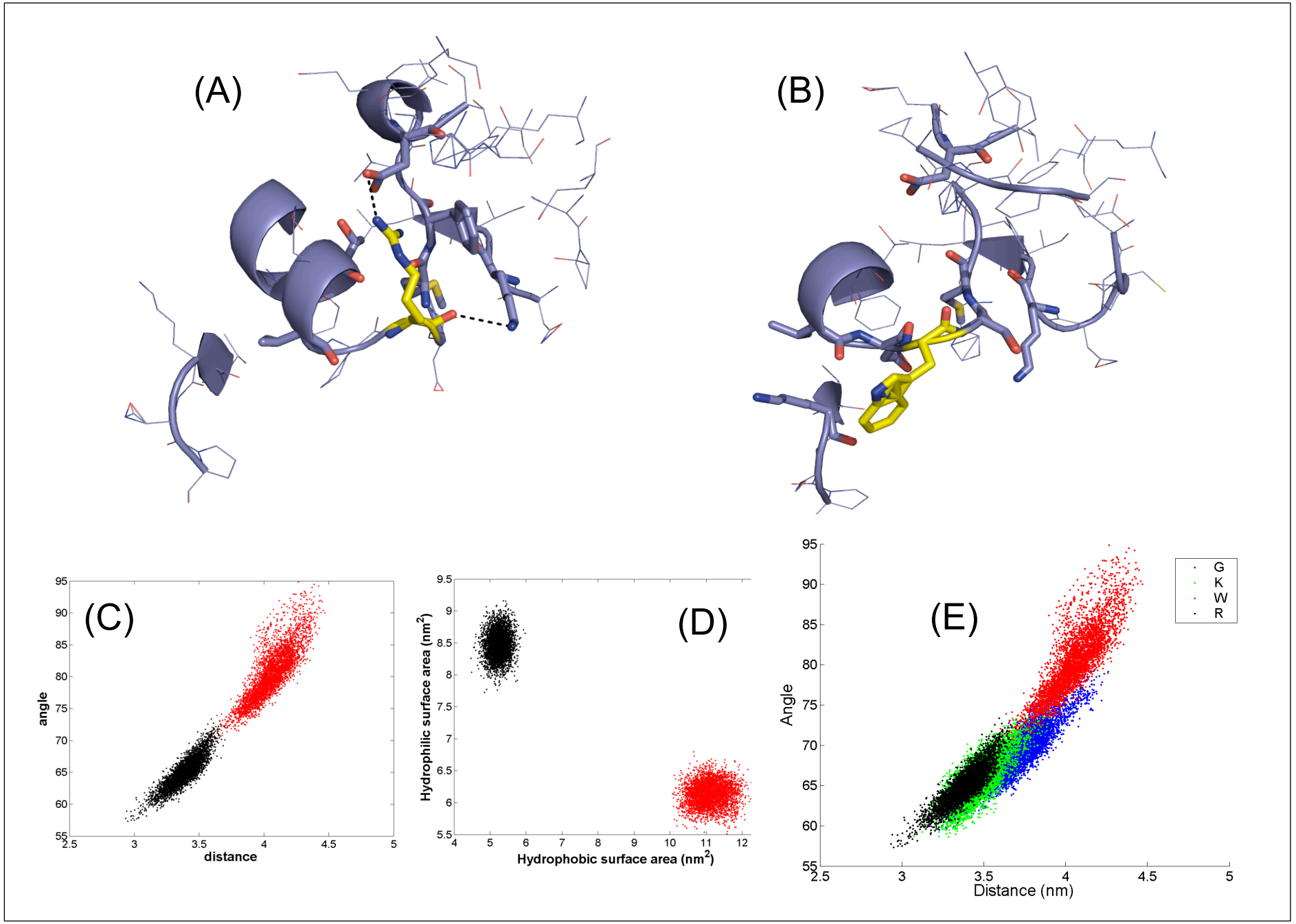

(A)
(B)
(C)
angle
distance
(D)
Hydrophilic surface area (nm^2)
Hydrophobic surface area (nm^2)
(E)
Angle
Distance (nm)
G
K
W
R

Fig. 7