was used to allow for covariates. We calculated the effect size (Cohen's *d*) for variables with a significant group difference.

Then, we investigated whether amino acids could serve as a diagnostic tool using a discriminant function analysis. In the logistic regression analysis, we calculated the correct classification rate. All analyses were performed with Stata/SE 10.0 software for windows (Stata Corp., College Station, TX).

Acknowledgments

We gratefully acknowledge the resources provided by a nonprofit organization, the Asperger Society of Japan, and participating families.

We also acknowledge Drs. Masanobu Kajizuka and Azusa Fujita for their technical assistance.

Author Contributions

Conceived and designed the experiments: CS SS KJT NT NM. Performed the experiments: CS SS KJT KH HM KI KM TW YK. Analyzed the data: CS SS KO NT. Contributed reagents/materials/analysis tools: KJT KM KS MT KN. Wrote the paper: CS SS NT.

References

1. Baron-Cohen S, Scott FJ, Allison C, Williams J, Bolton P, et al. (2009) Prevalence of autism-spectrum conditions: UK school-based population study. Br J Psychiatry 194: 500–509.
2. Volkmar FR, Pauls D (2003) Autism. Lancet 362: 1133–1141.
3. Blaylock RL (2008) A possible central mechanism in autism spectrum disorders, part 1. Altern Ther Health Med 14: 46–53.
4. Howlin P, Asgharian A (1999) The diagnosis of autism and Asperger syndrome: findings from a survey of 770 families. Developmental Medicine and Child Neurology 41: 834–839.
5. Olney JW (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science 164: 719–721.
6. Manev H, Favaron M, Guidotti A, Costa E (1989) Delayed increase of Ca2+ influx elicited by glutamate: role in neuronal death. Mol Pharmacol 36: 106–112.
7. Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int 51: 333–355.
8. Fatemi SH (2008) The hyperglutamatergic hypothesis of autism. Prog Neuropsychopharmacol Biol Psychiatry 32: 911, author reply 912–913.
9. Blaylock RL, Strunecka A (2009) Immune-Glutamatergic Dysfunction as a Central Mechanism of the Autism Spectrum Disorders. Current Medicinal Chemistry 16: 157–170.
10. Fatemi SH, Halt AR, Stary JM, Kanodia R, Schulz SC, et al. (2002) Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices. Biol Psychiatry 52: 805–810.
11. Page LA, Daly E, Schmitz N, Simmons A, Toal F, et al. (2006) In vivo 1H-magnetic resonance spectroscopy study of amygdala-hippocampal and parietal regions in autism. Am J Psychiatry 163: 2189–2192.
12. McGale EH, Pye IF, Stonier C, Hutchinson EC, Aber GM (1977) Studies of the inter-relationship between cerebrospinal fluid and plasma amino acid concentrations in normal individuals. J Neurochem 29: 291–297.
13. Alfredsson G, Wiesel FA, Tylec A (1988) Relationships between glutamate and monoamine metabolites in cerebrospinal fluid and serum in healthy volunteers. Biol Psychiatry 23: 689–697.
14. Rainesalo S, Keranen T, Palmio J, Peltola J, Oja SS, et al. (2004) Plasma and cerebrospinal fluid amino acids in epileptic patients. Neurochem Res 29: 319–324.
15. Miulli DE, Norwell DY, Schwartz FN (1993) Plasma concentrations of glutamate and its metabolites in patients with Alzheimer's disease. J Am Osteopath Assoc 93: 670–676.
16. Ilzecka J, Stelmasiak Z, Solski J, Wawrzycki S, Szpetnar M (2003) Plasma amino acids concentration in amyotrophic lateral sclerosis patients. Amino Acids 25: 69–73.
17. Shinohe A, Hashimoto K, Nakamura K, Tsujii M, Iwata Y, et al. (2006) Increased serum levels of glutamate in adult patients with autism. Prog Neuropsychopharmacol Biol Psychiatry 30: 1472–1477.
18. Moreno-Fuenmayor H, Borjas L, Arrieta A, Valera V, Socorro-Candanoza L (1996) Plasma excitatory amino acids in autism. Invest Clin 37: 113–128.
19. Arnold GL, Hyman SL, Mooney RA, Kirby RS (2003) Plasma amino acids profiles in children with autism: potential risk of nutritional deficiencies. J Autism Dev Disord 33: 449–454.
20. Aldred S, Moore KM, Fitzgerald M, Waring RH (2003) Plasma amino acid levels in children with autism and their families. J Autism Dev Disord 33: 93–97.
21. Croonenburghs J, Deboutte D, Maes M (2002) Pathophysiology of autism: current opinions. Acta Neuropsychiatrica 14: 93–102.
22. MMWR Surveill Summ (2007) Prevalence of autism spectrum disorders-autism and developmental disabilities monitoring network, six sites, United States 56: 11.
23. Lord C, Schopler E, Revicki D (1982) Sex differences in autism. J Autism Dev Disord 12: 317–330.
24. McLennan JD, Lord C, Schopler E (1993) Sex differences in higher functioning people with autism. J Autism Dev Disord 23: 217–227.
25. Schwarz E, Guest PC, Rahmoune H, Wang L, Levin Y, et al. (2010) Sex-specific serum biomarker patterns in adults with Asperger's syndrome. Mol Psychiatry.
26. Rolf LH, Haarmann FY, Grotemeyer KH, Kehrer H (1993) Serotonin and amino acid content in platelets of autistic children. Acta Psychiatr Scand 87: 312–316.
27. Bak LK, Schousboe A, Waagepetersen HS (2006) The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. J Neurochem 98: 641–653.
28. Hawkins RA, O'Kane RL, Simpson IA, Vina JR (2006) Structure of the blood-brain barrier and its role in the transport of amino acids. J Nutr 136: 218S–226S.
29. Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65: 1–105.
30. Hosoya K, Sugawara M, Asaba H, Terasaki T (1999) Blood-brain barrier produces significant efflux of L-aspartic acid but not D-aspartic acid: in vivo evidence using the brain efflux index method. J Neurochem 73: 1206–1211.
31. Brune CW, Kim SJ, Hanna GL, Courchesne E, Lord C, et al. (2008) Family-Based Association Testing of OCD-associated SNPs of SLC1A1 in an autism sample. Autism Res 1: 108–113.
32. Gadow KD, Roohi J, DeVincent CJ, Kirsch S, Hatchwell E (2010) Glutamate transporter gene (SLC1A1) single nucleotide polymorphism (rs301430) and repetitive behaviors and anxiety in children with autism spectrum disorder. J Autism Dev Disord 40: 1139–1145.
33. Kantojarvi K, Onkamo P, Vanhala R, Alen R, Hedman M, et al. (2010) Analysis of 9p24 and 11p12–13 regions in autism spectrum disorders: rs1340513 in the JMJD2C gene is associated with ASDs in Finnish sample. Psychiatr Genet 20: 102–108.
34. Ortinski PI, Dong J, Mungenast A, Yue C, Takano H, et al. (2010) Selective induction of astrocytic gliosis generates deficits in neuronal inhibition. Nat Neurosci 13: 584–591.
35. Laurence JA, Fatemi SH (2005) Glial fibrillary acidic protein is elevated in superior frontal, parietal and cerebellar cortices of autistic subjects. Cerebellum 4: 206–210.
36. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 57: 67–81.
37. Pais TF, Figueiredo C, Peixoto R, Braz MH, Chatterjee S (2008) Necrotic neurons enhance microglial neurotoxicity through induction of glutaminase by a MyD88-dependent pathway. J Neuroinflammation 5: 43.
38. Association AP (2000) Diagnostic and Statistical Manual of Mental Disorders, 4th ed. Text Revision. Washington DC.
39. Lord CRM, 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: 27.
40. Ohsawa S (1998) saishin rinsho kagaku kensa hou. Medical Technology 26: 7.
41. Yao JK, Dougherty GG, Jr., Reddy RD, Keshavan MS, Montrose DM, et al. (2010) Altered interactions of tryptophan metabolites in first-episode neuroleptic-naive patients with schizophrenia. Mol Psychiatry 15: 938–953.

Association of Transcription Factor Gene *LMX1B* with Autism

Ismail Thanseem[1☯], Kazuhiko Nakamura[1*☯], Ayyappan Anitha[2☯], Shiro Suda[3☯], Kazuo Yamada[4], Yoshimi Iwayama[4], Tomoko Toyota[4], Masatsugu Tsujii[2,5], Yasuhide Iwata[1], Katsuaki Suzuki[2], Hideo Matsuzaki[2], Keiko Iwata[2], Toshiro Sugiyama[6,7], Takeo Yoshikawa[4], Norio Mori[1,2]

1 Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan, 2 Research Center for Child Mental Development, Hamamatsu University School of Medicine, Hamamatsu, Japan, 3 Department of Psychiatry, Jichi Medical University, Shimotsuke, Japan, 4 Laboratory of Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan, 5 Faculty of Sociology, Chukyo University, Toyota, Aichi, Japan, 6 Aichi Children's Health and Medical Center, Obu, Aichi, Japan, 7 Department of Child and Adolescent Psychiatry, Hamamatsu University School of Medicine, Hamamatsu, Japan

Abstract

Multiple lines of evidence suggest a serotoninergic dysfunction in autism. The role of *LMX1B* in the development and maintenance of serotoninergic neurons is well known. In order to examine the role, if any, of *LMX1B* with autism pathophysiology, a trio-based SNP association study using 252 family samples from the AGRE was performed. Using pair-wise tagging method, 24 SNPs were selected from the HapMap data, based on their location and minor allele frequency. Two SNPs (rs10732392 and rs12336217) showed moderate association with autism with p values 0.018 and 0.022 respectively in transmission disequilibrium test. The haplotype AGCGTG also showed significant association ($p = 0.008$). Further, *LMX1B* mRNA expressions were studied in the postmortem brain tissues of autism subjects and healthy controls samples. *LMX1B* transcripts was found to be significantly lower in the anterior cingulate gyrus region of autism patients compared with controls ($p = 0.049$). Our study suggests a possible role of *LMX1B* in the pathophysiology of autism. Based on previous reports, it is likely to be mediated through a seretoninergic mechanism. This is the first report on the association of *LMX1B* with autism, though it should be viewed with some caution considering the modest associations we report.

Citation: Thanseem I, Nakamura K, Anitha A, Suda S, Yamada K, et al. (2011) Association of Transcription Factor Gene *LMX1B* with Autism. PLoS ONE 6(8): e23738. doi:10.1371/journal.pone.0023738

Editor: Kenji Hashimoto, Chiba University Center for Forensic Mental Health, Japan

Received June 27, 2011; **Accepted** July 22, 2011; **Published** August 25, 2011

Copyright: © 2011 Thanseem et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to Dr. K. Nakamura. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: nakamura@hama-med.ac.jp

☯ These authors contributed equally to this work.

Introduction

Autism and other developmental disabilities, clinically referred to as autism spectrum disorders (ASDs), are characterized by impairments in communication skills and social interaction, and the presence of repetitive stereotyped behaviors and interests. It is typically diagnosed by the age of three and has a prevalence rate of 60-70 per 10,000 children in broader diagnostic criteria as per the most recent estimates [1]. ASDs are considered to be among the most heritable of all psychiatric disorders. A recent largest population based twin study comprised of 10,895 twin pairs, reported 80% heritability for ASDs [2], confirming the previously reported heritability estimates [3,4]. Linkage, candidate gene and whole genome association studies have suggested several genes and chromosomal regions associated with the disorder. However, none of these known causes individually account for more than 1–2% of the cases, and specific genetic mechanisms underlying the heritability of the disorder still remain largely cryptic. It was found that many different genetic changes in unrelated genes can cause indistinguishable ASD features; this genetic heterogeneity necessitate the need to look for more potential candidate genes associated with the disorder.

The LIM homeodomain transcription factor 1b (LMX1B) was initially characterized as a key regulator of the normal dorsoventral patterning in the developing limbs [5]. Several mutations reported in this gene have been found to lead to the pleiotropic phenotype, the nail patella syndrome [6–8]. Later, the role of *Lmx1b* in the development and maintenance of serotoninergic (5HTergic) neurons in the central nervous system (CNS) was reported, and thereafter, underlying mechanisms were studied in detail. *Lmx1b* knock-out mice were found to be lacking the entire central 5HTergic neurons [9,10]. Further, it was shown that overexpression of *Lmx1b* enhances differentiation of mouse embryonic stem cells into 5HT neurons [11]. In addition to its role in the development of central 5HTeregic neurons, *Lmx1b* is also required for the normal biosynthesis of 5HT in adult brain, and possibly for the regulation of normal functions of 5HTergic neurons [12].

A role of 5HTergic system in the pathophysiology of autism was proposed based on following observations, a) hyperserotonemia in the whole blood cells and platelets of 25–50% of patients with autism [13,14], b) depletion of tryptophan, the 5HT precursor, in ASD patients increased some stereotype behaviors associated with the disorder [15], c) treatment with selective serotonin reuptake inhibitors has shown to be effective in ameliorating the repetitive and/or

compulsive behaviors in some autistic individuals [16] and d) recent neuroimaging studies have shown low levels of brain 5HT synthesis in autistic children [17] and reduction in serotonin transporter (SLC6A4) binding in different brain regions of both children and adults with the disorder [18,19]. Compliant with these reports, several genetic association studies involving genes in the 5HT metabolism with a focus on the *SLC6A4* were also attempted. While several *SLC6A4* polymorphisms were shown to be associated with the disorder in some studies [20,21], others failed to replicate the findings [22].

Taking together, these results provide compelling, though inconsistent evidence for the role of 5HTergic system in the pathophysiologic mechanism of ASDs. In view of the importance of LMX1B in the development of 5-HTergic neurons, it would be interesting to study its role in autism. Here we performed a trio-based study to examine the association of *LMX1B* with autism. We also assessed any alterations in the expression *LMX1B* in the postmortem brain samples of autism patients as compared to healthy controls.

Results

Single SNP TDT

Mendelian inheritance inconsistencies were not observed for any of the SNPs. For each SNP, >99% of the genotypes were scored; none of the SNPs showed deviation from HWE.

The results of TDT analysis are shown in Table 1. rs10732392 ($p = 0.018$; OR = 1.764; 95% CI for OR 1.095–2.842) and rs12336217 ($p = 0.022$; OR = 1.748; 95% CI for OR 1.076–2.841) showed significant associations with autism. However, these associations did not withstand the multiple testing correction. Overtransmission was observed for the minor allele A (62.82%) of rs10732392 and for minor allele G (62.67%) of rs12336217.

LD analysis

LD analysis based on D' values identified six distinct haploblocks across *LMX1B* gene. The first block consists of SNPs 01 to 06, the second block SNPs 08 and 09, the third block 10 and 11, fourth block 12 to 16, fifth block 18 and 19 and the sixth block included SNPs 20 to 22 (Figure 1).

Haplotype TDT

The results of haplotype TDT is given in Table 2. Based on the LD structure of *LMX1B*, associations of haplotypes in the six haploblocks were analysed. The haplotype AGCGTG of the first block showed significant association with autism ($p = 0.008$).

LMX1B expression in the postmortem brains

No significant difference in age, sex and postmortem intervals was observed between autism and control groups in all the brain

Table 1. Single SNP TDT results of *LMX1B* SNPs in 252 trio samples.

Marker	db SNP ID	Genomic Location	Variation*	Location	Minor allele frequency†	T (%)‡	*p*-value§
SNP 1	rs10732392	129396037	G:A	Intron 2	0.078	48.92	***0.018***
SNP 2	rs10760444	129396434	A:G	Intron 2	0.449	48.23	0.214
SNP 3	rs10448285	129397014	C:T	Intron 2	0.376	50.64	0.601
SNP 4	rs12336217	129399870	A:G	Intron 2	0.075	48.98	***0.022***
SNP 5	rs7858338	129406644	T:C	Intron 2	0.26	51.61	0.085
SNP 6	rs11793373	129407543	G:A	Intron 2	0.252	50.6	0.513
SNP 7	rs10819190	129408513	G:A	Intron 2	0.414	49.56	0.739
SNP 8	rs6478750	129409198	T:C	Intron 2	0.408	49.91	0.948
SNP 9	rs12555734	129411242	C:A	Intron 2	0.24	51.25	0.16
SNP 10	rs13285227	129413298	C:T	Intron 2	0.348	49.11	0.439
SNP 11	rs944103	129413490	G:A	Intron 2	0.472	49.05	0.526
SNP 12	rs12555176	129414303	G:T	Intron 2	0.074	50.11	0.809
SNP 13	rs7854658	129414938	G:A	Intron 2	0.21	50.57	0.486
SNP 14	rs10987386	129416317	C:T	Intron 2	0.191	49.5	0.519
SNP 15	rs12551234	129417809	G:C	Intron 2	0.407	49.92	0.949
SNP 16	rs7853174	129419990	G:A	Intron 2	0.394	49.04	0.452
SNP 17	rs10819194	129422023	G:A	Intron 2	0.422	51.78	0.189
SNP 18	rs4322101	129428677	A:G	Intron 2	0.416	51.19	0.37
SNP 19	rs7030919	129438872	A:G	Intron 2	0.115	49.49	0.37
SNP 20	rs3737048	129458092	G:T	Intron 6	0.107	50.39	0.474
SNP 21	rs10987413	129459438	G:A	3′	0.333	50.65	0.56
SNP 22	rs10760450	129459628	C:T	3′	0.21	50.58	0.475
SNP 23	rs10733682	129460914	G:A	3′	0.486	51.27	0.41
SNP 24	rs4083644	129461714	C:T	3′	0.28	49.93	0.943

T: Transmitted.
*Common allele is listed first.
†Based on the parental genotypes of 252 trios.
‡T% of common allele is listed, § Computed on the basis of likelihood ratio test; significant p-values (<0.05) are indicated in bold italics.
doi:10.1371/journal.pone.0023738.t001

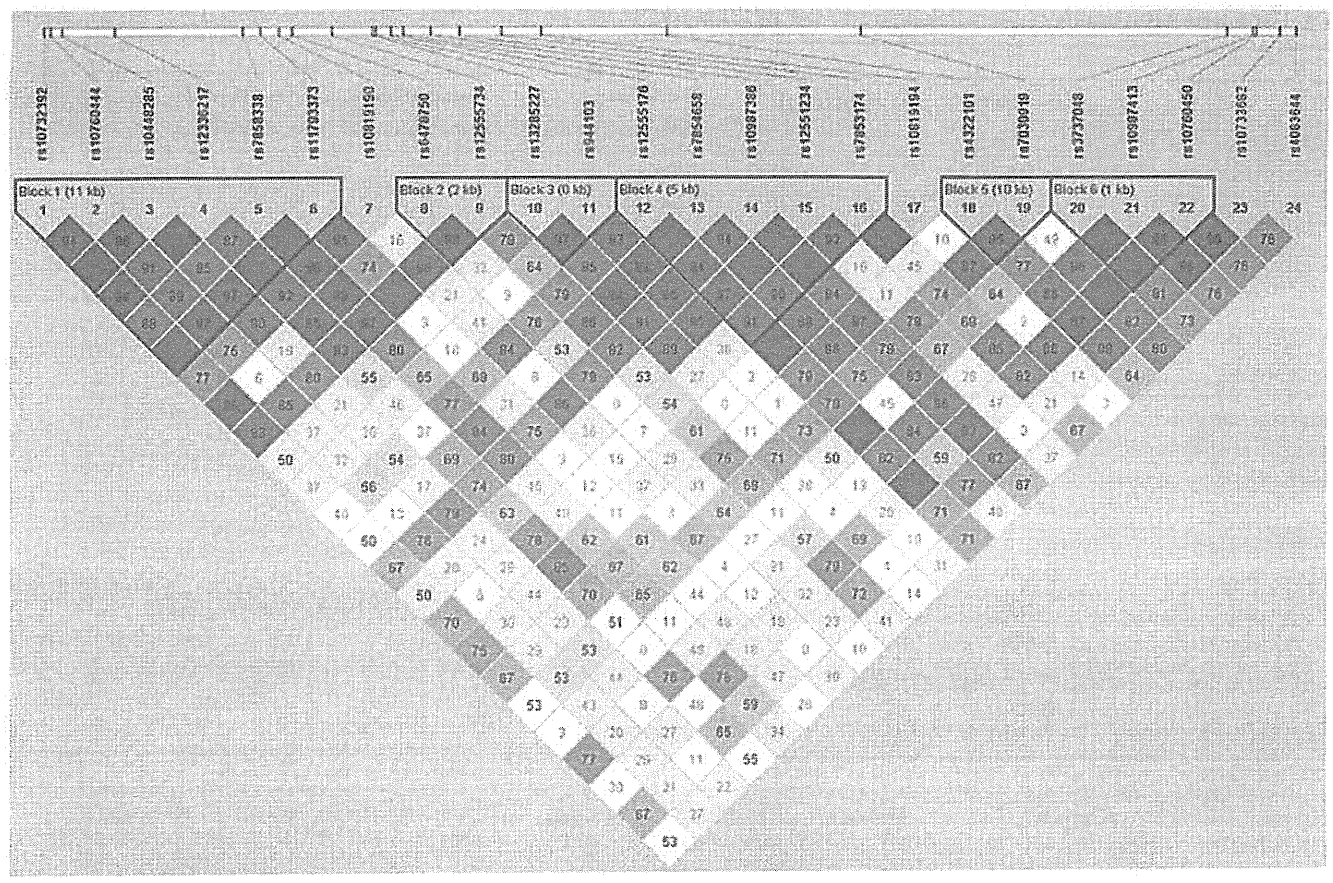

Figure 1. Haploblock structure of *LMX1B*. Six haplotype blocks were identified based on D' values calculated from 252 trios.
doi:10.1371/journal.pone.0023738.g001

regions (ACG, MC and THL). There was a significant difference in *LMX1B* expression between the autism and control group in the ACG ($p = 0.049$) (Figure 2). Expression was significantly lower in autism groups with a fold change of ($2^{-\Delta\Delta C_T}$) 0.43. No *LMX1B* expression could be detected in the other two brain regions (MC and TH).

Discussion

In this study, we examined the association of the transcription factor gene *LMX1B* with autism in Caucasian population. In the trio-based study, we found nominal associations for two SNPs (rs10732392 and rs12336217) and a haplotype with autism. To the best of our knowledge, this is the first study which reported an association between *LMX1B* and autism; a previous study reported the association between *LMX1B* and schizophrenia [23], which is also a neurodevelopmental disorder. Both the SNPs which are found to be associated with the disorder are located in the introns (intron 2) and may lack any direct functional importance. We also found that the *LMX1B* mRNA expression in general, is rather low in adult brain; detected only in ACG. However, *LMX1B* mRNAs were found to be significantly lower in the ACG of autistic brains than the similar regions of control brain tissues.

Multiple lines of evidence suggested a serotoninergic dysfunction in many patients with autism, although the results are still inconclusive. Involvement of several transcription factors are reported in the 5HTergic differentiation. In mammalian CNS, a sequential activation of transcription factors in the hindbrain, starting with the regulation of the expression of *Nkx2-2* by the *Shh* signaling pathway, has been proposed [9]. It was observed that 5HT neurons are absent in the mice lacking *Nkx2-2* [24]. It occupies the highest hierarchical position in the genetic cascade that involved in the development of 5HT neurons. Another transcription factor *Pet1*, expressed in the post mitotic 5HT neurons was reported to be the terminal differentiation factor, which acts in the final step of the transcriptional cascade that establishes the final identity of 5HT neurons. Mice lacking *Pet1* had 70–80% fewer 5-HT neurons than normal mice. The *Lmx1b* ablation does not affect the expression Nkx2.2 and Shh [9,25] putting these factors upstream of Lmx1b. However, during development, *Lmx1b* precedes *pet1*, and *Lmx1b* knock-out mice showed loss of *Pet1* expression [10]. *In vivo*, *Pet1* expression was increased in neurons overexpressing *Lmx1b* [11]. Thus, *Lmx1b* has been proposed as an essential link between Nkx2.2 and Pet1 in the genetic cascade that controls the early specification and terminal differentiation of 5HTergic neurons in the hindbrain. Lmx1b expression was shown to be the rate limiting step in this cascade of events for specifying the 5HT phenotype [11]. Further, Lmx1b, together with Pet1, is also involved in the serotonin metabolism as it controls a set of molecules essential for the serotonin synthesis (TPH2), vesicular transport (VMAT2) and reuptake after synaptic release (SLC6A4) in the developing as well as adult brain [10,12].

ACG region plays important role in the pathophysiology of autism as shown by previous reports [26,27]. Our positron emission tomography studies had shown that a reduction in SLC6A4 binding in the cingulate cortices is associated with an impairment of social cognition in autistic subjects [19]. The present finding of reduced *LMX1B* expression in the ACG of

Table 2. Haplotype associations of SNPs belonging to the six LD blocks of *LMX1B*, in 252 trios.

Block	Haplotype*	Frequency	T(%)	Individual *p*-value†	Permutation *p*-value‡	Block *p*-value
Block 1 (SNPs 01–06)	GGTATG	0.355	51.67	0.6291	1	
	GACATA	0.25	48.81	0.7487	1	
	GACACG	0.244	45.71	0.2568	0.994	
	AGCGTG	0.073	66.13	*0.0079*	0.114	
	GACATG	0.052	51.42	0.8461	1	
	GGTACG	0.014	30.77	0.1658	0.97	0.086
Block 2 (SNPs 08–09)	CC	0.406	50.23	0.9432	1	
	TC	0.353	54.03	0.2242	0.987	
	TA	0.239	44.23	0.1255	0.892	0.258
Block 3 (SNPs 10–11)	CG	0.525	48.4	0.6123	1	
	TA	0.345	52.71	0.4094	1	
	CA	0.126	48.79	0.8046	1	0.731
Block 4 (SNPs 12–16)	GGCGA	0.379	53.41	0.3114	0.998	
	GGCGG	0.209	45.31	0.2362	0.991	
	GACCG	0.201	48.99	0.8072	1	
	GGTCG	0.119	55.41	0.2624	0.994	
	TGTCG	0.071	48.81	0.8455	1	0.595
Block 5 (SNPs 18–19)	AA	0.58	52.42	0.4476	1	
	GA	0.304	47.25	0.1587	0.966	
	GG	0.112	53.61	0.4772	1	0.354
Block 6 (SNPs 20–22)	GGC	0.35	55.39	0.111	0.868	
	GAC	0.332	48.19	0.59	1	
	GGT	0.21	47.63	0.5365	1	
	TGC	0.107	46.45	0.4947	1	0.512

T: Transmitted / (Transmitted + Untransmitted).
‡10,000 permutations.
*All possible combinations of haplotypes with frequency >0.01 †Significant *p*-values (<0.05) are indicated in bold italics.
doi:10.1371/journal.pone.0023738.t002

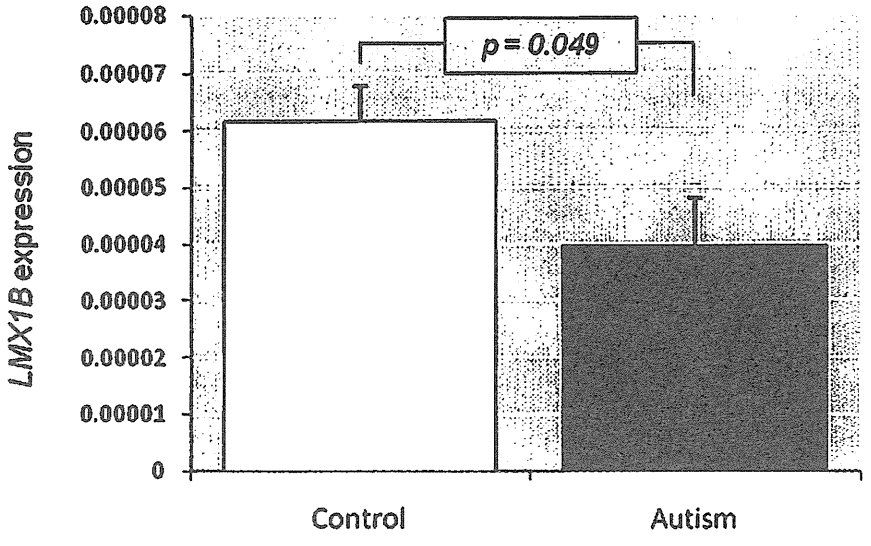

Figure 2. *LMX1B* expression in the brain. *LMX1B* expression in the anterior cingulate gyrus region of the brain of autism patients compared to that of control samples.
doi:10.1371/journal.pone.0023738.g002

Table 3. Postmortem brain tissue information.

Sample ID[a]	Diagnosis	Age (years)	Gender	PMI (hours)	Race	Cause of death	Brain regions[b]
UMB 818	Control	27	M	10	Caucasian	Multiple injuries	ACG
UMB 1065	Control	15	M	12	Caucasian	Multiple injuries	ACG, THL
UMB 1297	Control	15	M	16	African American	Multiple injuries	ACG, MC, THL
UMB 1407	Control	9	F	20	African American	Asthma	ACG, MC, THL
UMB 1541	Control	20	F	19	Caucasian	Head injuries	ACG, MC, THL
UMB 1649	Control	20	M	22	Hispanic	Multiple injuries	ACG, MC, THL
UMB 1708	Control	8	F	20	African American	Asphyxia, multiple injuries	ACG, MC, THL
UMB 1790	Control	13	M	18	Caucasian	Multiple injuries	ACG
UMB 1793	Control	11	M	19	African American	Drowning	ACG, MC, THL
UMB 1860	Control	8	M	5	Caucasian	Cardiac Arrhythmia	ACG
UMB 4543	Control	28	M	13	Caucasian	Multiple injuries	ACG, MC, THL
UMB 4638	Control	15	F	5	Caucasian	Chest injuries	ACG
UMB 4722	Control	14	M	16	Caucasian	Multiple injuries	ACG, MC, THL
UMB 797	Autism	9	M	13	Caucasian	Drowning	ACG, THL
UMB 1638	Autism	20	F	50	Caucasian	Seizure	ACG, MC, THL
UMB 4231	Autism	8	M	12	African American	Drowning	ACG, MC, THL
UMB 4721	Autism	8	M	16	African American	Drowning	ACG, MC, THL
UMB 4899	Autism	14	M	9	Caucasian	Drowning	ACG, MC, THL
B 5000	Autism	27	M	8.3	NA	NA	ACG, MC, THL
B 6294	Autism	16	M	NA	NA	NA	ACG, MC, THL
B 6640	Autism	29	F	17.83	NA	NA	ACG, MC, THL

[a]Autism Tissue Program (ATP) identifier.
[b]Brain regions for which, each sample was available.
M: Male; F: Female, PMI: Postmortem interval, ACG: Anterior cingulate gyrus; MC: Motor cortex; THL: Thalamus; NA: Not available.
doi:10.1371/journal.pone.0023738.t003

autism group, therefore, could have some deleterious effects on the serotonergic system, given the role of LMX1B in the differentiation of 5HT neurons in developing brain, and in the maintenance of 5HT system in adult brain.

In conclusion, we report a possible association of the transcription factor *LMX1B* with autism pathogenesis. However, our results should be interpreted with some caution, given the limitations in sample size of postmortem brain samples and the modest associations we found in genetic and gene expression studies.

Materials and Methods

Subjects

DNA samples from trio families recruited to the Autism Genetic Resource Exchange [28] were used for the single nucleotide polymorphism (SNP) association study. We selected 252 trios families with male offspring scored for autism. Only Caucasians (white) were selected and non-idiopathic autism cases were excluded.

Brain samples

Frozen postmortem brain tissues from autistic patients and controls were provided by the Autism Tissue Program (ATP; Princeton, NJ; http://www.autismtissueprogram.org) and Harvard Brain Tissue Research Center (HBTRC; Belmont, MA; http://www.brainbank.mclean.org/). Tissues were obtained from three brain regions important in cognitive and behavior processing namely a) anterior cingulate gyrus (ACG- 8 autism and 13 controls), b) motor cortex (MC- 7 autism and 8 controls), and c) thalamus (THL-8 autism and 9 controls). The demographic features of the samples are described in Table 3.

Selection of SNPs

LMX1B, located in 9q33.3 (129,376,748 – 129,463,311), is 86.56kb in size and consists of eight exons. The genomic structure is based on the UCSC (http://www.genome.ucsc.edu) assembly of the human genome. SNPs for the association studies were selected using the information from international HapMap project (http://www.hapmap.org) and National Centre for Biotechnology Information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP). On the basis of their genomic locations and minor allele frequencies (MAF >0.1), 24 SNPs were selected (Figure 3; Table 1), using the pair-wise tagging option of Haploview.v4.1 (http://www.broad.mit.edu/mpg/haploview).

Genotyping

Assay-on-demand/Assay-by-design SNP genotyping products (ABI, Foster City, CA, USA) were used to score SNPs, based on the TaqMan assay method [29]. Genotypes were determined in ABI PRISM 7900HT Sequence Detection System (SDS) (Applied Biosystems), and analyzed using SDS v2.0 (ABI).

Statistical Analysis

PedCheck v1.1 (http://www.watson.hgen.pitt.edu) was used to identify and eliminate all Mendelian inheritance inconsistencies in

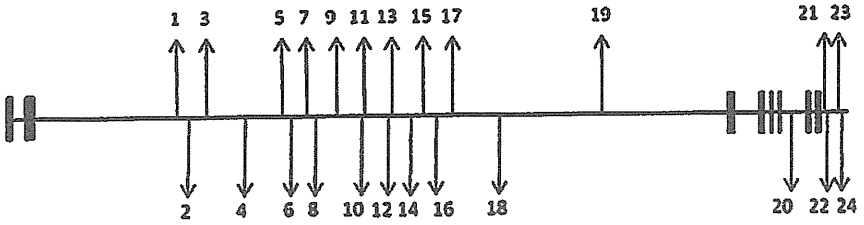

Figure 3. Genomic structure of *LMX1B* gene. Locations of SNPs selected for the association study, based on the HapMap data on Caucasian population, are denoted by arrows. Exons are indicated by boxes.
doi:10.1371/journal.pone.0023738.g003

the trio genotype data. SNPs were tested for Hardy–Weinberg Equilibrium (HWE) using Haploview. SNP associations were examined by transmission disequilibrium test (TDT), using the TDTPHASE option of UNPHASED v2.403 (http://portal.litbio.org); expectation maximization (EM) algorithm was used to resolve uncertain haplotypes, to infer missing genotypes and to provide maximum-likelihood estimation of frequencies.

A linkage disequilibrium (LD) plot was constructed using the D' values. Pair-wise LD values between SNPs were estimated using Haploview. Subsequently, associations of haplotypes (frequency >0.01) belonging to the various haploblocks of *LMX1B* were also examined using Haploview.

Extraction of RNA from brain tissues

The brain tissues were homogenized by ultrasonication and total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's protocol. The RNA samples were further purified using RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions. The quantity (absorbance at 260 nm) and quality (ratio of absorbance at 260 nm and 280 nm) of RNA were estimated with a NanoDrop ND-1000 Spectrophotometer (Scrum, Tokyo, Japan).

Quantitative real-time reverse transcriptase PCR (qRT-PCR)

ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) was used to synthesize first-strand cDNA from the total RNA according to the manufacturer's protocol.

RT-PCR primers for *LMX1B* (NM_001174146.1) (F-cctttgagcaagtaaggataatgaatg, R-gggactgaatttcccagcaa) and endogenous reference *GAPDH* (NM_002046.3) (F-atcagcaatgcctcctgcac, R-tggcatggactgtggtcatg) were designed using primer express v2.0 (Applied Biosystems). SYBR Green qRT-PCR assays were performed using QuantiTect SYBR Green PCR kit (Qiagen). All the reactions were performed in triplicate, in the ABI PRISM 7900HT Sequence Detection System. C_T values, which reflect the mRNA expression levels, were determined. *LMX1B* C_T of each sample was normalized to the corresponding C_T for the internal control by calculating ΔC_T (ΔC_T = Target gene C_T – *GAPDH* C_T) to obtain the relative mRNA expression of the target gene. Quantification of the gene expression was performed by calculating $\Delta\Delta C_T$ ($\Delta\Delta C_T$ = ΔC_T of the autistic group - ΔC_T of the control group).The fold change in gene expression between the two groups was determined by calculating $2^{-\Delta\Delta C_T}$.

Statistical analysis

For the gene expression studies, statistical calculations were performed using PSAW statistics 18.0 software (IBM-SPSS, Tokyo, Japan). The difference in age and postmortem interval between autistic and control groups was examined by t-test. The chi-square test was used to examine the sex distribution; alteration in gene expression between the two groups was analyzed by Mann-Whitney U-test.

Acknowledgments

We thank Dr. Jane Pickett, Director of Brain Resources and Data, Autism Tissue Program, for facilitating brain tissue collection. Human tissue was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland. Tissue samples were also provided by the Harvard Brain Tissue Resource Center, which is supported in part by PHS grant number R 24 MH 068855. We thank Ms. Tae Takahashi for technical assistance.

Author Contributions

Conceived and designed the experiments: IT KN AA SS NM. Performed the experiments: IT AA SS. Analyzed the data: IT AA AA KY Y. Iwayama TY. Contributed reagents/materials/analysis tools: TT MT Y. Iwata KS HM KI TS TY. Wrote the paper: IT KN AA NM.

References

1. Fombonne E, Quirke S, Hagen A (2009) Prevalence and interpretation of recent trends in rates of pervasive developmental disorders. Mcgill J Med 12: 73.
2. Lichtenstein P, Carlstrom E, Rastam M, Gillberg C, Anckarsater H (2010) The genetics of autism spectrum disorders and related neuropsychiatric disorders in childhood. Am J Psychiatry 167: 1357–1363.
3. Bailey A, Le Couteur A, Gottesman I, Bolton P, Simonoff E, et al. (1995) Autism as a strongly genetic disorder: evidence from a British twin study. Psychol Med 25: 63–77.
4. Steffenburg S, Gillberg C, Hellgren L, Andersson L, Gillberg IC, et al. (1989) A twin study of autism in Denmark, Finland, Iceland, Norway and Sweden. J Child Psychol Psychiatry 30: 405–416.
5. Johnson RL, Tabin CJ (1997) Molecular models for vertebrate limb development. Cell 90: 979–990.
6. Knoers NV, Bongers EM, van Beersum SE, Lommen EJ, van Bokhoven H, et al. (2000) Nail-patella syndrome: identification of mutations in the LMX1B gene in Dutch families. J Am Soc Nephrol 11: 1762–1766.
7. Milla E, Herman I, Gamundi MJ, Martinez-Gimeno M, Carballo M (2007) Novel LMX1B mutation in familial nail-patella syndrome with variable expression of open angle glaucoma. Mol Vis 13: 639–648.
8. Marini M, Bocciardi R, Gimelli S, Di Duca M, Divizia MT, et al. (2010) A spectrum of LMX1B mutations in Nail-Patella syndrome: new point mutations, deletion, and evidence of mosaicism in unaffected parents. Genet Med 12: 431–439.
9. Ding YQ, Marklund U, Yuan W, Yin J, Wegman L, et al. (2003) Lmx1b is essential for the development of serotonergic neurons. Nat Neurosci 6: 933–938.
10. Zhao ZQ, Scott M, Chiechio S, Wang JS, Renner KJ, et al. (2006) Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. J Neurosci 26: 12781–12788.
11. Doimazon V, Alenina N, Markossian S, Mancip J, van de Vrede Y, et al. (2011) Forced expression of LIM homeodomain transcription factor 1b enhances

differentiation of mouse embryonic stem cells into serotonergic neurons. Stem Cells Dev 20: 301–311.
12. Song NN, Xiu JB, Huang Y, Chen JY, Zhang L, et al. (2011) Adult raphe-specific deletion of Lmx1b leads to central serotonin deficiency. PLoS One 6: e15998.
13. Cook EH, Leventhal BL (1996) The serotonin system in autism. Curr Opin Pediatr 8: 348–354.
14. Anderson GM, Gutknecht L, Cohen DJ, Brailly-Tabard S, Cohen JH, et al. (2002) Serotonin transporter promoter variants in autism: functional effects and relationship to platelet hyperserotonemia. Mol Psychiatry 7: 831–836.
15. McDougle CJ, Naylor ST, Cohen DJ, Aghajanian GK, Heninger GR, et al. (1996) Effects of tryptophan depletion in drug-free adults with autistic disorder. Arch Gen Psychiatry 53: 993–1000.
16. Kolevzon A, Mathewson KA, Hollander E (2006) Selective serotonin reuptake inhibitors in autism: a review of efficacy and tolerability. J Clin Psychiatry 67: 407–414.
17. Chandana SR, Behen ME, Juhasz C, Muzik O, Rothermel RD, et al. (2005) Significance of abnormalities in developmental trajectory and asymmetry of cortical serotonin synthesis in autism. Int J Dev Neurosci 23: 171–182.
18. Makkonen I, Riikonen R, Kokki H, Airaksinen MM, Kuikka JT (2008) Serotonin and dopamine transporter binding in children with autism determined by SPECT. Dev Med Child Neurol 50: 593–597.
19. Nakamura K, Sekine Y, Ouchi Y, Tsujii M, Yoshikawa E, et al. (2010) Brain serotonin and dopamine transporter bindings in adults with high-functioning autism. Arch Gen Psychiatry 67: 59–68.
20. McCauley JL, Olson LM, Dowd M, Amin T, Steele A, et al. (2004) Linkage and association analysis at the serotonin transporter (SLC6A4) locus in a rigid-compulsive subset of autism. Am J Med Genet B Neuropsychiatr Genet 127B: 104–112.
21. Sutcliffe JS, Delahanty RJ, Prasad HC, McCauley JL, Han Q, et al. (2005) Allelic heterogeneity at the serotonin transporter locus (SLC6A4) confers susceptibility to autism and rigid-compulsive behaviors. Am J Hum Genet 77: 265–279.
22. Ramoz N, Reichert JG, Corwin TE, Smith CJ, Silverman JM, et al. (2006) Lack of evidence for association of the serotonin transporter gene SLC6A4 with autism. Biol Psychiatry 60: 186–191.
23. Bergman O, Westberg L, Nilsson LG, Adolfsson R, Eriksson E (2010) Preliminary evidence that polymorphisms in dopamine-related transcription factors LMX1A, LMX1B and PITX3 are associated with schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 34: 1094–1097.
24. Pattyn A, Vallstedt A, Dias JM, Sander M, Ericson J (2003) Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. Development 130: 4149–4159.
25. Cheng L, Chen CL, Luo P, Tan M, Qiu M, et al. (2003) Lmx1b, Pet-1, and Nkx2.2 coordinately specify serotonergic neurotransmitter phenotype. J Neurosci 23: 9961–9967.
26. Haznedar MM, Buchsbaum MS, Metzger M, Solimando A, Spiegel-Cohen J, et al. (1997) Anterior cingulate gyrus volume and glucose metabolism in autistic disorder. Am J Psychiatry 154: 1047–1050.
27. Ohnishi T, Matsuda H, Hashimoto T, Kunihiro T, Nishikawa M, et al. (2000) Abnormal regional cerebral blood flow in childhood autism. Brain 123(Pt9): 1838–1844.
28. Geschwind DH, Sowinski J, Lord C, Iversen P, Shestack J, et al. (2001) The autism genetic resource exchange: a resource for the study of autism and related neuropsychiatric conditions. Am J Hum Genet 69: 463–466.
29. Ranade K, Chang MS, Ting CT, Pei D, Hsiao CF, et al. (2001) High-throughput genotyping with single nucleotide polymorphisms. Genome Res 11: 1262–1268.

Iwata et al. Molecular Autism 2011, 2:16
http://www.molecularautism.com/content/2/1/16

Molecular Autism

SHORT REPORT Open Access

Investigation of the serum levels of anterior pituitary hormones in male children with autism

Keiko Iwata[1†], Hideo Matsuzaki[1*†], Taishi Miyachi[1], Chie Shimmura[1], Shiro Suda[1], Kenji J Tsuchiya[1], Kaori Matsumoto[1], Katsuaki Suzuki[1], Yasuhide Iwata[2], Kazuhiko Nakamura[2], Masatsugu Tsujii[1,3], Toshirou Sugiyama[4], Kohji Sato[5] and Norio Mori[1,2]

Abstract

Background: The neurobiological basis of autism remains poorly understood. The diagnosis of autism is based solely on behavioural characteristics because there are currently no reliable biological markers. To test whether the anterior pituitary hormones and cortisol could be useful as biological markers for autism, we assessed the basal serum levels of these hormones in subjects with autism and normal controls.

Findings: Using a suspension array system, we determined the serum levels of six anterior pituitary hormones, including adrenocorticotropic hormone and growth hormone, in 32 drug-naive subjects (aged 6 to 18 years, all boys) with autism, and 34 healthy controls matched for age and gender. We also determined cortisol levels in these subjects by enzyme-linked immunosorbent assay. Serum levels of adrenocorticotropic hormone, growth hormone and cortisol were significantly higher in subjects with autism than in controls. In addition, there was a significantly positive correlation between cortisol and adrenocorticotropic hormone levels in autism.

Conclusion: Our results suggest that increased basal serum levels of adrenocorticotropic hormone accompanied by increased cortisol and growth hormone may be useful biological markers for autism.

Introduction

Autism is a neurodevelopmental disorder, categorised as a pervasive developmental disorder, and is characterised by severe and sustained impairment in social interaction, by deviance in communication, and patterns of behaviour and interest. The aetiology of autism is not well understood, although it is thought to involve genetic, immunologic and environmental factors [1]. The diagnosis of autism is based solely on behavioural characteristics, as there is currently no biological marker for autism.

Several studies have examined anterior pituitary hormones as possible biological markers for autism [2-8]. The anterior pituitary gland synthesises and secretes adrenocorticotropic hormone (ACTH), growth hormone (GH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and prolactin (PRL). Of these hormones, ACTH deserves special attention, because it is the hormone involved in the hypothalamic-pituitary-adrenal (HPA) axis, which may be affected in autism [3,4,6,9-11]. The HPA axis is the basis for emotion and social interaction, through the synthesis and/or release of corticotropin-releasing hormone, ACTH and cortisol. All previous studies that have measured basal ACTH levels in autism have shown an increase in the serum/plasma levels of this hormone [3,4,6,10], except for one study that showed no difference [7]. Unlike the results for ACTH, the results for serum cortisol levels in autism are inconsistent, with studies reporting either no difference between patients and controls [3,5-7] or a decrease in patients [4,10]. With regard to the basal serum/plasma levels of other anterior pituitary hormones in autism spectrum disorders (ASDs), the results are again contradictory: a decrease in patients [7] or no difference from controls [4] for GH; a decrease in patients [12,13] or no difference from controls [7] for FSH; and no difference from controls for TSH and PRL [2,4,7].

The conflicting findings in the measurement of anterior pituitary hormones in ASDs probably arise because of differences in the subject population. For instance, many

* Correspondence: matsu@hama-med.ac.jp
† Contributed equally
[1]Research Center for Child Mental Development, Hamamatsu University School of Medicine, Hamamatsu, Japan
Full list of author information is available at the end of the article

BioMed Central

© 2011 Iwata et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

studies used samples from both male and female patients; however, a recent systemic serum proteome profiling study pointed out that male and female patients with Asperger's disorder had distinct biomarker fingerprints [7]. Moreover, the secretion of anterior pituitary hormones may be modified by antipsychotic and antiepileptic medications [14-16], which often had not been taken into consideration in previous studies.

In this study, we assessed the basal concentrations of anterior pituitary hormone and cortisol in serum from male, drug-naïve subjects with autism.

Methods

Ethics approval

This study was approved by the ethics committee of the Hamamatsu University School of Medicine. All participants and their guardians were given a complete description of the study, and provided written informed consent before enrolment.

Subjects

In total, 32 boys with autism (aged 6 to 18 years) and 34 healthy controls matched for agen and gender participated in this study. All the participants were Japanese, born and living in the Aichi, Gifu or Shizuoka prefectures of central Japan.

Based on interviews and available records, including those from hospitals, the diagnosis of autism were made based on the *Diagnostic and Statistical Manual, Fourth Revision, Text Revision* (DSM-IV-TR) criteria. The Autism Diagnostic Interview-Revised (ADI-R) was also conducted by two of the authors (KJT and KM), both of whom are experienced and reliable at diagnosing autism with the Japanese version of the ADI-R. We also used the Wechsler Intelligence Scale for Children, Third Edition, to evaluate the intelligence quotient. Comorbid psychiatric illnesses were excluded by means of the Structured Clinical Interview for DSM-IV (SCID). Participants were excluded from the study if they had any symptoms of inflammation, a diagnosis of fragile X syndrome, epileptic seizures, obsessive-compulsive disorder, affective disorder, or any additional psychiatric or neurological diagnosis. All the autistic subjects were drug-naive, and were not taking any dietary supplements.

Healthy control subjects were recruited locally by an advertisement. All control subjects underwent a comprehensive assessment of their medical history to eliminate individuals with any neurological or other medical disorders. The SCID was also conducted to scrutinise any personal or family history of past or present mental illness. None of the control subjects initially recruited fulfilled any of these exclusion criteria.

Fasting blood samples were collected by venipuncture from all participants between 11.00 and 12.30 hours, and the samples were kept at room temperature for 30 minutes. The samples were then separated by centrifugation, divided into aliquots of 200 μl, and stored at -80°C until use. Serum levels of anterior pituitary hormones were assayed using a suspension array system (Bio-Plex; Bio-Rad, Hercules, CA, USA), with a panel of pituitary antibodies (Milliplex MAP Human Pituitary Panel; Millipore, Billerica, MA, USA). This system allows simultaneous identification of pituitary hormones with antibodies chemically attached to fluorescently labelled microbeads. The beads were resuspended in assay buffer, and the reaction mixture was quantified using a protein array reader (Bio-Plex; Bio-Rad). Serum levels of cortisol were determined using a commercially available sandwich ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Clinical characteristics (age, weight, height and body mass index (BMI)) were analysed using an unpaired *t*-test, after confirmation that there were no significant differences in variance as assessed by the *F*-test. Comparisons of concentrations of anterior pituitary hormones and cortisol between subjects with autism and controls were made using the Mann-Whitney *U*-test. In these multiple comparisons, a Bonferroni-adjusted nominal *P*-value threshold of 0.007 was used. Evaluation of the relationships between serum hormone levels and clinical variables or symptom profiles, and those between hormone levels, was performed using Spearman's rank correlation coefficient. Additionally, linear regression analyses were conducted to examine whether any change in the hormone levels could be accounted for by another variable, such as age or the levels of other hormones. Values of $P < 0.05$ were considered significant. All statistical analyses were performed using SPSS software (version 12.0 J; IBM, Tokyo, Japan).

Results

The characteristics of all the participants are summarised in Table 1. There were no significant differences in the distributions of age, weight, height or BMI between the autism group and the control group.

The serum levels of ACTH were 11.6 ± 5.1 pg/mL in subjects with autism and 7.2 ± 3.1 pg/mL in controls. Therefore, the level of ACTH in subjects with autism was significantly higher than that in controls ($U = 185.0$, $P < 0.001$, by Mann-Whitney *U*-test) (Table 1, Figure 1A). The serum levels of GH in subjects with autism (6495.4 ± 9072.2 pg/mL) were also significantly higher than those in controls (1590.1 ± 2447.5 pg/mL; $U = 305.0$, $P = 0.002$, Mann-Whitney *U*-test) (Table 1, Figure 1B). We carried out regression analyses to test the effect of age and other hormones on GH levels, because these

Table 1 Clinical characteristics of the normal controls and subjects with autism[a]

	Control group (n = 34)	Autism group (n = 32)	*P*-value
Age, years	12.4 ± 2.6 (6 to 18)	12.3 ± 3.2 (6 to 18)	NS
Weight, kg	42.3 ± 14.3 (15.6 to 89.3)	41.8 ± 15.0 (17.5 to 96.6)	NS
Height, cm	150.8 ± 14.8 (111 to 174)	148.7 ± 17.9 (110 to 178)	NS
BMI, kg/m^2	18.1 ± 3.5 (12.7 to 32.4)	18.3 ± 3.1 (13.9 to 30.5)	NS
ADI-R			
Domain A score	-	20.2 ± 4.9 (10 to 27)	-
Domain BV score	-	13.6 ± 3.9 (8 to 21)	-
Domain C score	-	5.4 ± 2.0 (3 to 9)	-
Domain D score	-	3.1 ± 1.0 (2 to 5)	-
WISC-III			
Verbal IQ	-	91.3 ± 21.6 (48 to 133)	-
Performance IQ	-	95.3 ± 21.1 (47 to 131)	-
Full-scale IQ	-	91.0 ± 23.2 (44 to 134)	-
Anterior pituitary hormones			
ACTH, pg/mL	7.2 ± 3.1 (3.7 to 14.2)	11.6 ± 5.1 (3.7 to 26.3)	< 0.001
GH, pg/mL	1590.1 ± 2447.5 (34.8 to 13708.0)	6495.4 ± 9072.2 (30.7 to 34811.5)	0.002
FSH, mIU/mL	3.8 ± 2.0 (0.8 to 8.1)	5.7 ± 3.7 (0.6 to 16.4)	NS
LH, mIU/mL	1.4 ± 1.7 (0.1 to 7.1)	2.5 ± 2.5 (0.1 to 11.8)	NS
TSH, μIU/mL	3.6 ± 1.4 (1.0 to 7.4)	3.3 ± 2.2 (0.3 to 11.2)	NS
PRL, ng/mL	20.9 ± 9.1 (4.4 to 38.0)	25.2 ± 14.0 (8.2 to 66.9)	NS
Cortisol, ng/mL	58.3 ± 25.3 (16.8 to 116.8)	74.2 ± 20.0 (23.5 to 101.5)	0.004

[a]Values are expressed as mean ± SD(range).

Abbreviations: BMI, body mass index; ADI-R, Autism Diagnostic Interview-Revised; WISC-III, the third edition of the Wechsler Intelligence Scale for Children; IQ, intelligence quotient; ACTH, adrenocorticotropic hormone; GH, growth hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; PRL, prolactin; NS, not significant.

may affect GH levels [17-19]. After controlling for age and measured hormone levels (FSH, LH, TSH, PRL, ACTH and cortisol), we confirmed a significant difference in GH levels ($F_{(1,63)}$ = 9.504, P = 0.003 for age; $F_{(1,63)}$ = 7.238, P = 0.009 for FSH; $F_{(1,63)}$ = 8.429, P = 0.005 for LH; $F_{(1,63)}$ = 9.891, P = 0.003 for TSH; $F_{(1,63)}$ = 9.033, P = 0.004 for PRL; $F_{(1,62)}$ = 6.611, P = 0.013 for ACTH; and $F_{(1,60)}$ = 4.687, P = 0.034 for cortisol) between subjects with autism and controls. There were no significant differences in FSH, LH, TSH or PRL levels between autistic and control subjects (Table 1).

The serum levels of cortisol were 74.2 ± 20.0 ng/mL in subjects with autism and 58.3 ± 25.3 ng/mL in controls. Therefore, the level of cortisol in subjects with autism was significantly higher than that in controls (U = 289.0, P = 0.004, Mann-Whitney U-test) (Table 1, Figure 1C). There was a significantly positive correlation between cortisol and ACTH levels in subjects with autism (r_s = 0.562, P < 0.001, Spearman's rank correlation coefficient) (Figure 1D). We also examined the correlations between serum ACTH, GH and cortisol levels and the symptom profiles in subjects with autism. The ADI-R domain A, BV, and C scores were used as the symptom profiles. There were no significant correlations between the levels of any of the hormones and the symptom profiles (data not shown).

Discussion

We found that serum levels of ACTH and cortisol in subjects with autism were significantly higher than those in healthy controls. When the relationship between the levels of ACTH and cortisol was examined in subjects with autism, the levels of ACTH were significantly and positively correlated with the levels of cortisol, suggesting that in autism, cortisol secretion may be upregulated by increasing ACTH through the HPA axis [20]. It is possible that people with autism respond to the stress of venipuncture with activation of the HPA axis, leading to the elevation of ACTH; however, in this study, we found that the venipuncture effect was the same in autistic and control subjects, and therefore the observed differences are more likely to be due to the pathology of autism than to acute stress.

An increase in ACTH levels in people with autism is the most consistent result reported from studies of anterior pituitary hormones [3,4,6,10]. In functional imaging studies of the limbic system, which is the neural basis of emotions and social interactions, people with autism have been shown to have impaired circuitry in extinguishing fear responses [21]. Because the limbic system influences the HPA axis [22], the abnormal levels of ACTH and cortisol may be due to alterations in limbic system function [8,23].

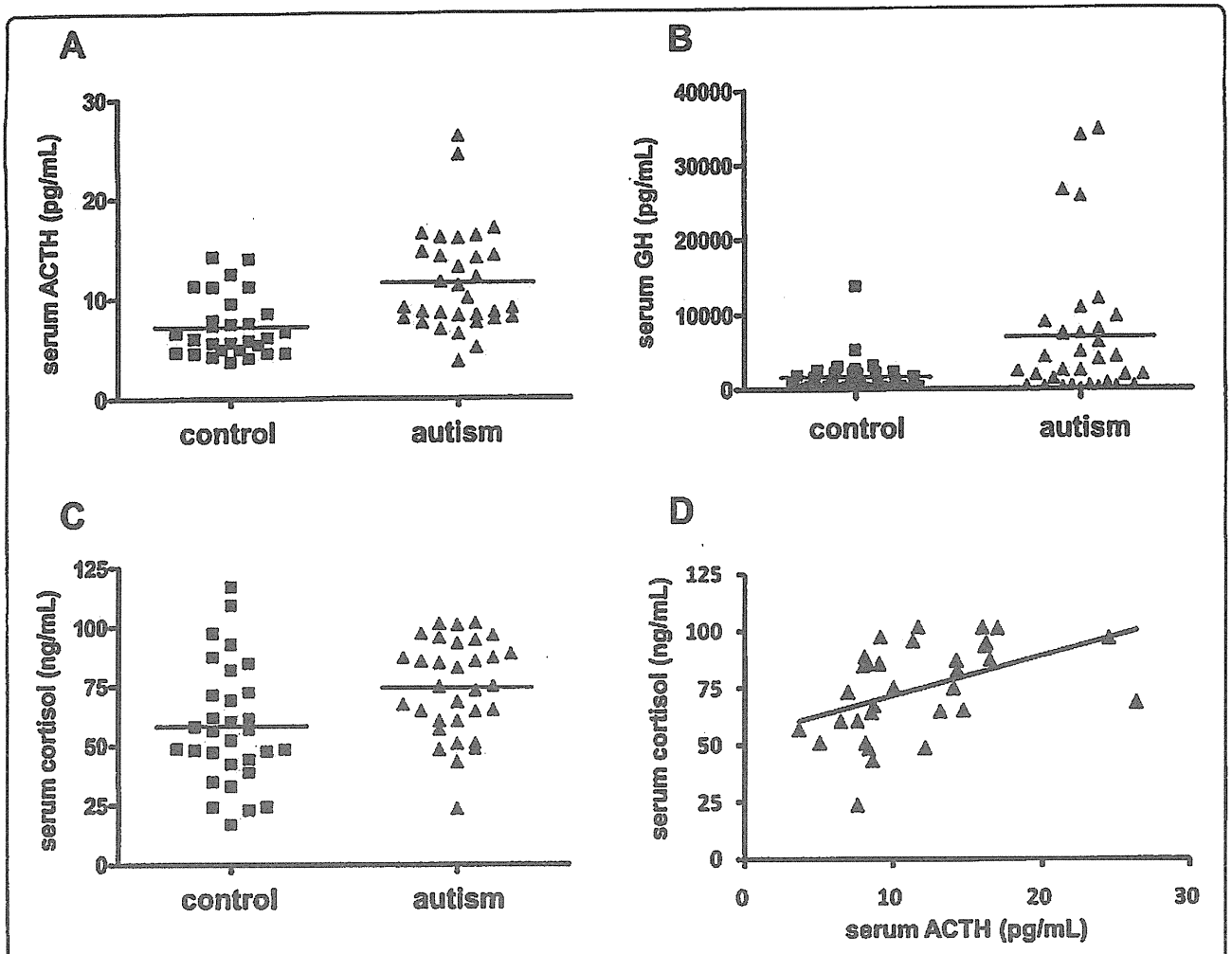

Figure 1 Serum levels of adrenocorticotropic hormone (ACTH), growth hormone (GH) and cortisol in normal controls and children with autism. (A) The serum levels of ACTH in subjects with autism (n = 32) were significantly higher ($P < 0.001$, Mann-Whitney *U*-test) than those in normal controls (n = 34). Two autistic subjects had very high values, but there were no apparent differences in clinical parameters between these subjects and the others. (B) The serum levels of GH in subjects with autism (n = 32) were significantly higher ($P = 0.002$, Mann-Whitney *U*-test) than those in normal controls (n = 34). Four autistic subjects (which did not include the two with high ACTH) had very high GH values; again, there were no overt differences in clinical features between these four subjects and the others. (C) The serum levels of cortisol in subjects with autism (n = 32) were significantly higher ($P = 0.004$, Mann-Whitney *U*-test) than those in normal controls (n = 34). (D) Correlation between serum cortisol levels and ACTH levels in subjects with autism. There was a positive correlation ($P < 0.001$, Spearman's $\rho_s = 0.562$) between these hormone levels.

Unlike the present study, in which we found high levels of cortisol in subjects with autism, previous studies have reported low or no overt change in cortisol levels in autism [3-7,10]. Cortisol levels can be modified by psychotropic medications [14,15]; we recruited drug-naive subjects in this study, and all the previous studies [3,5,6], except one [4], have used drug-free subjects to examine cortisol levels. Therefore, it is unlikely that the discrepancy between the present and previous results arose because of differences in the medication status of the participants. This, in turn, suggests that an alternative explanation is required. The present study included only male subjects, whereas previous studies comprised both male and female subjects [3-6,10]. In addition, the age range of the participants of the present study (6 to 18 years) was different from that of some of the previous studies, which enrolled adults only [5-7]. Furthermore, we collected the blood samples at around midday, whereas previous studies used samples collected in the morning [3-6,10]. Gender [7], age [24] and sampling time [24] are all known to be important factors influencing the cortisol level.

We also found that serum levels of GH in subjects with autism were significantly higher than those in healthy controls. There are no available data to interpret this increased GH in basal conditions in autism. However,

because the serum levels of glutamate have been shown to be increased in adults with autism [25], and because intravenous administration of excitatory amino acids stimulates GH secretion [26-28], the increased basal GH levels in autism seen in our study may, at least in part, be due to a high concentration of glutamate in the circulation.

In this study, we found no significant correlations between cortisol levels and autistic symptoms as assessed by the ADI-R. This is in contrast to the results of Hamza *et al.* [10], who found an inverse correlation between hormone-stimulated plasma cortisol levels and the severity of autistic symptoms as assessed by the Childhood Autism Rating Scale. This discrepancy may be caused by the different scales used for the evaluation of clinical features.

There are some limitations to our study. The small sample size renders the data presented here preliminary. In addition, the study included only male participants. A larger study with subjects of both genders will be necessary, although separate analysis may still be warranted to eliminate the confounding effect of gender on hormone levels.

Conclusion

Our results suggest that increased basal serum levels of ACTH accompanied by increased cortisol and GH may be useful biological markers for autism.

List of abbreviations

ACTH: adrenocorticotropic hormone; ADI-R: Autism Diagnostic Interview-Revised; FSH: follicle-stimulating hormone; GH: growth hormone; HPA: hypothalamic-pituitary-adrenal axis; LH: lutelnizing hormone; PRL: prolactin; SCID: Structured Clinical Interview for DSM-IV; TSH: thyroid-stimulating hormone.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to HM), a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to NM) and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to KI).

Author details

[1]Research Center for Child Mental Development, Hamamatsu University School of Medicine, Hamamatsu, Japan. [2]Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan. [3]Faculty of Contemporary Sociology, Chukyo University, Toyota, Japan. [4]Department of Child and Adolescent Psychiatry, Hamamatsu University School of Medicine, Hamamatsu, Japan. [5]Department of Anatomy and Neuroscience, Hamamatsu University School of Medicine, Hamamatsu, Japan.

Authors' contributions

HM, KI, KSat and NM designed this study. KN, MT and TS were involved in the recruitment of participants. HM, TM and KN collected blood samples. KTJ and KM conducted clinical evaluations. KI, HM, CS, SS and YI measured and analysed serum levels of hormones from the anterior pituitary gland. KI, HM, KSuz, KSat and NM participated in manuscript preparation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 9 April 2011 Accepted: 19 October 2011
Published: 19 October 2011

References

1. Volkmar FR, Lord C, Bailey A, Schultz RT, Klin A: Autism and pervasive developmental disorders. *J Child Psychol Psychiatry* 2004, 45:135-170.
2. Cohen DJ, Young JG, Lowe TL, Harcherik D: Thyroid hormone in autistic children. *J Autism Dev Disord* 1980, 10:445-450.
3. Tordjman S, Anderson G, McBride P, Hertzig M, Snow M, Hall L, Thompson S, Ferrari P, Cohen D: Plasma beta-endorphin, adrenocorticotropin hormone, and cortisol in autism. *J Child Psychol Psychiatry* 1997, 38:705-715.
4. Curin JM, Terzić J, Petković ZB, Zekan L, Terzić IM, Susnjara IM: Lower cortisol and higher ACTH levels in individuals with autism. *J Autism Dev Disord* 2003, 33:443-448.
5. Strous RD, Golubchik P, Maayan R, Mozes T, Tuati-Werner D, Weizman A, Spivak B: Lowered DHEA-S plasma levels in adult individuals with autistic disorder. *Eur Neuropsychopharmacol* 2005, 15:305-309.
6. Tani P, Lindberg N, Matto V, Appelberg B, Nieminen-von Wendt T, von Wendt L, Porkka-Heiskanen T: Higher plasma ACTH levels in adults with Asperger syndrome. *J Psychosom Res* 2005, 58:533-536.
7. Schwarz E, Guest PC, Rahmoune H, Wang L, Levin Y, Ingudomnukul E, Ruta L, Kent L, Spain M, Baron-Cohen S, Bahn S: Sex-specific serum biomarker patterns in adults with Asperger's syndrome. *Mol Psychiatry* 2010.
8. Spratt EG, Nicholas JS, Brady KT, Carpenter LA, Hatcher CR, Meekins KA, Furlanetto RW, Charles JM: Enhanced cortisol response to stress in children in autism. *J Autism Dev Disord* 2011.
9. Corbett BA, Mendoza S, Wegelin JA, Carmean V, Levine S: Variable cortisol circadian rhythms in children with autism and anticipatory stress. *J Psychiatry Neurosci* 2008, 33:227-234.
10. Hamza RT, Hewedi DH, Ismail MA: Basal and adrenocorticotropic hormone stimulated plasma cortisol levels among Egyptian autistic children: relation to disease severity. *Ital J Pediatr* 2010, 36:71.
11. Jansen LM, Gispen-de Wied CC, van der Gaag RJ, van Engeland H: Differentiation between autism and multiple complex developmental disorder in response to psychosocial stress. *Neuropsychopharmacology* 2003, 28:582-590.
12. Geier DA, Geier MR: A clinical and laboratory evaluation of methionine cycle-transsulfuration and androgen pathway markers in children with autistic disorders. *Horm Res* 2006, 66:182-188.
13. Geier DA, Geier MR: A prospective assessment of androgen levels in patients with autistic spectrum disorders: biochemical underpinnings and suggested therapies. *Neuro Endocrinol Lett* 2007, 28:565-573.
14. Leskiewicz M, Budziszewska B, Lason W: Endocrine effects of antiepileptic drugs. *Przegl Lek* 2008, 65:795-798.
15. Levy AD, Van de Kar LD: Endocrine and receptor pharmacology of serotonergic anxiolytics, antipsychotics and antidepressants. *Life Sci* 1992, 51:83-94.
16. Madhusoodanan S, Parida S, Jimenez C: Hyperprolactinemia associated with psychotropics--a review. *Hum Psychopharmacol* 2010, 25:281-297.
17. Holsboer F: Psychiatric implications of altered limbic-hypothalamic-pituitary-adrenocortical activity. *Eur Arch Psychiatry Neurol Sci* 1989, 238:302-322.
18. Mauras N, Rogol AD, Haymond MW, Veldhuis JD: Sex steroids, growth hormone, insulin-like growth factor-1: neuroendocrine and metabolic regulation in puberty. *Horm Res* 1996, 45:74-80.
19. Zachmann M: Assessment of growth hormone secretion in children. *Keio J Med* 1990, 39:173-186.
20. Jacobson L: Hypothalamic-pituitary-adrenocortical axis regulation. *Endocrinol Metab Clin North Am* 2005, 34:271-292, vii.
21. Sweeten TL, Posey DJ, Shekhar A, McDougle CJ: The amygdala and related structures in the pathophysiology of autism. *Pharmacol Biochem Behav* 2002, 71:449-455.
22. Dallman MF, Akana SF, Strack AM, Scribner KS, Pecoraro N, La Fleur SE, Houshyar H, Gomez F: Chronic stress-induced effects of corticosterone on brain: direct and indirect. *Ann N Y Acad Sci* 2004, 1018:141-150.

23. Corbett BA, Schupp CW, Levine S, Mendoza S: Comparing cortisol, stress, and sensory sensitivity in children with autism. *Autism Res* 2009, 2:39-49.
24. Hardy R, Cooper MS: Adrenal gland and bone. *Arch Biochem Biophys* 2010, 503:137-145.
25. Shinohe A, Hashimoto K, Nakamura K, Tsujii M, Iwata Y, Tsuchiya KJ, Sekine Y, Suda S, Suzuki K, Sugihara G, *et al*: Increased serum levels of glutamate in adult patients with autism. *Prog Neuropsychopharmacol Biol Psychiatry* 2006, 30:1472-1477.
26. Estienne MJ, Schillo KK, Green MA, Hileman SM, Boling JA: N-methyl-d, l-aspartate stimulates growth hormone but not luteinizing hormone secretion in the sheep. *Life Sci* 1989, 44:1527-1533.
27. Gay VL, Plant TM: N-methyl-D,L-aspartate elicits hypothalamic gonadotropin-releasing hormone release in prepubertal male rhesus monkeys (Macaca mulatta). *Endocrinology* 1987, 120:2289-2296.
28. Shahab M, Nusser KD, Griel LC, Deaver DR: Effect of a single intravenous injection of N-methyl-D,L-aspartic acid on secretion of luteinizing hormone and growth hormone in Holstein bull calves. *J Neuroendocrinol* 1993, 5:469-473.

doi:10.1186/2040-2392-2-16
Cite this article as: Iwata *et al*: Investigation of the serum levels of anterior pituitary hormones in male children with autism. *Molecular Autism* 2011 2:16.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

BioMed Central

OPEN ACCESS Freely available online

PLoS one

Plasma Cytokine Profiles in Subjects with High-Functioning Autism Spectrum Disorders

Katsuaki Suzuki[1]*, Hideo Matsuzaki[1], Keiko Iwata[1], Yosuke Kameno[2], Chie Shimmura[2], Satomi Kawai[3], Yujiro Yoshihara[4], Tomoyasu Wakuda[2], Kiyokazu Takebayashi[1], Shu Takagai[2], Kaori Matsumoto[1], Kenji J. Tsuchiya[1], Yasuhide Iwata[2], Kazuhiko Nakamura[2], Masatsugu Tsujii[5], Toshirou Sugiyama[6], Norio Mori[1,2]

1 Research Center for Child Mental Development, Hamamatsu University School of Medicine, Hamamatsu, Japan, 2 Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan, 3 Department of Psychiatry, National Hospital Organization Tenryu Hospital, Hamamatsu, Japan, 4 Department of Psychiatry, Koujin Hospital, Nagoya, Japan, 5 Faculty of Sociology, Chukyo University, Toyota, Japan, 6 Department of Child and Adolescent Psychiatry, Hamamatsu University School of Medicine, Hamamatsu, Japan

Abstract

Background: Accumulating evidence suggests that dysregulation of the immune system is involved in the pathophysiology of autism spectrum disorders (ASD). The aim of the study was to explore immunological markers in peripheral plasma samples from non-medicated subjects with high-functioning ASD.

Methodology/Principal Findings: A multiplex assay for cytokines and chemokines was applied to plasma samples from male subjects with high-functioning ASD (n = 28) and matched controls (n = 28). Among a total of 48 analytes examined, the plasma concentrations of IL-1β, IL-1RA, IL-5, IL-8, IL-12(p70), IL-13, IL-17 and GRO-α were significantly higher in subjects with ASD compared with the corresponding values of matched controls after correction for multiple comparisons.

Conclusion/Significance: The results suggest that abnormal immune responses as assessed by multiplex analysis of cytokines may serve as one of the biological *trait* markers for ASD.

Citation: Suzuki K, Matsuzaki H, Iwata K, Kameno Y, Shimmura C, et al. (2011) Plasma Cytokine Profiles in Subjects with High-Functioning Autism Spectrum Disorders. PLoS ONE 6(5): e20470. doi:10.1371/journal.pone.0020470

Editor: Takeo Yoshikawa, Rikagaku Kenkyūsho Brain Science Institute, Japan

Received April 6, 2011; **Accepted** April 26, 2011; **Published** May 27, 2011

Copyright: © 2011 Suzuki et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to KS (#22390221). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: k-suzuki@hama-med.ac.jp

Introduction

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by pervasive abnormalities in social interaction and communication, and repetitive and restricted behavioral patterns and interests. ASD include autistic disorder, Asperger's disorder and pervasive developmental disorder, not otherwise specified [1]. Susceptibility to ASD is clearly attributable to genetic factors [2], but the etiology of ASD is unknown, and no biomarkers have yet been proven to be characteristic of ASD.

Accumulating evidence suggests that dysregulation of the immune system may be implicated in the pathophysiology of ASD [3,4]. For instance, postmortem studies have shown that the protein levels of tumor necrosis factor α (TNF-α) and interleukin (IL)-6 [5], as well as the number of activated microglia [6], are significantly increased in the brains of subjects with ASD compared to controls. In addition, lipopolysaccharide-stimulated productions of TNF-α and IL-6 have been shown to be greater in peripheral blood mononuclear cells from subjects with ASD than those from controls [7]. And increased levels of inflammatory cytokines have been detected even in peripheral samples such as serum [8–13] or plasma [14–18] of patients with ASD. These findings suggest that the pattern of plasma cytokine levels could serve as a useful biological marker of ASD. However, the results of the previous studies addressing serum or plasma levels of cytokines in ASD appear to be inconsistent, probably due to variations in the experimental designs, diagnostic criteria used and age ranges of the subjects, although another possible explanation is that these inconsistencies reflect the heterogeneity of the ASD themselves.

Recent advances in multiplex technologies have enabled measurement of multiple analytes simultaneously. Multiplexing provides data on a large number of analytes, even when the sample volumes are limited [19,20]. In this study, we used a multiplex assay to measure a series of 48 cytokines in plasma samples from subjects with high-functioning ASD in comparison with matched control subjects. A recent systemic serum proteome profiling study reported that males and females with Asperger's disorder have distinct biomarker fingerprints [11]. Therefore, to prevent any potential confounding effect of sex, we recruited only males in this study. Also, cytokine profiles were only determined in the ASD male subjects who were more than 6 years of age, because a multiplex analysis of cytokines in plasma samples obtained from children less than 5 years of age (the majority of whom were males) was recently reported [14].

Results

Subjects

The characteristics of all participants are summarized in Table 1. There was no significant difference in the distribution of age ($t = 0.26$, $P = 0.79$) or full IQ ($t = 0.46$, $P = 0.65$) between the two groups, indicating that the subject matching was successful. Several pro-inflammatory cytokines, including TNF-α and IL-6, are known to be produced by adipose tissue, and the plasma levels of these cytokines have been correlated with parameters of obesity [21]. Therefore, we measured the weight and height of all the participants, and the body mass index (BMI) was calculated. There were no significant inter-group differences in the weight, height, or BMI. In subjects with ASD, 21 subjects with ASD were diagnosed with autistic disorder and the remaining 7 were considered to have PDD-NOS, according to the Autism Diagnostic Interview-Revised (ADI-R) [22].

Plasma levels of cytokines and chemokines by multiplex assay kits

The comparison of cytokine and chemokine detection is summarized in Table 2. Among a total of 48 analytes, plasma concentrations of IL-2, IL-15, basic FGF, GM-CSF and LIF did not reach the detection range in either group, and these five analytes were excluded from further analyses. Plasma levels of IL-1β, IL-1RA, IL-5, IL-8, IL-12(p70), IL-13, IL-17 and GRO-α were significantly higher in subjects with ASD compared with the corresponding values of matched controls after correction for multiple comparisons. Plasma levels of IL-4, IL-7, G-CSF, IFN-γ, MIP-1β, PDGF-BB, TNF-α, HGF and VEGF tended to be greater in the ASD group than in the control groups, but after correction for multiple comparisons, the differences did not reach the level of statistical significance. The mean levels of fold changes of the cytokines that differed significantly between the two groups are summarized in Figure 1.

We then examined the correlations between plasma levels of IL-1β, IL-1RA, IL-5, IL-12(p70), IL-13, IL-17 and GRO-α and clinical variables in the subjects with ASD. There were no statistically significant correlations between the plasma levels of analytes and clinical variables, including age, weight, height, BMI, IQ (full, verbal and performance) and severities in autistic symptoms as assessed by the ADI-R. When correlation coefficients were evaluated among the 7 analytes that showed significant elevation in ASD, there were significant correlations between IL-1β and IL-1RA (Pearson's $r = .626$, $P < 0.001$), between IL-5 and IL-13 ($r = .497$, $P = 0.007$), between IL-13 and IL-12(p70) ($r = .747$, $P < 0.001$) and between IL-8 and GRO-α ($r = .415$, $P = 0.028$).

Discussion

In the present study, plasma levels of IL-1β, IL-1RA, IL-5, IL-8, IL-12(p70), IL-13, IL-17 and GRO-α in the high-functioning male subjects with ASD were significantly higher than those of carefully matched control subjects. Our participants with ASD showed no signs or symptoms implying inflammatory diseases and were similar in parameters of obesity, including BMI, to controls. Thus, it is likely that the elevations in plasma levels of those analytes were significantly associated with the diagnosis of ASD. These results are in line with the studies mentioned above, which reported altered immune responses in individuals with ASD [3,4]. The fold changes of each analyte, however, ranged from 1.5 to 2.5, which values were far lower than those of inflammatory or autoimmune diseases. In addition, none of the analyte plasma levels were correlated with the severity of autistic symptoms. Therefore, it was suggested that the elevation of cytokines observed here may represent an abnormal steady-state immune response in subjects with ASD, and that such a multiplex analysis of cytokines may serve as one of the biological trait markers for the disorder.

Plasma levels of IL-1β and IL-1RA were elevated in ASD

IL-1β is a pro-inflammatory cytokine produced by various sources, including monocytes, macrophages, dendritic cells, neutrophil leukocytes and endothelial cells [23]. Among previous reports, two studies that examined serum levels of selected cytokines, i.e., IL-1 [12] and IL-1β [24], in autistic subjects reported no change. However, two other recent studies using multiplex assay in ASD have demonstrated a significant increase in plasma IL-1β levels in 2- to 5-year-old children with ASD [14] or in serum IL-1β levels in adults with Asperger's syndrome [11]. Given the wide variety of functions of IL-1β as an important mediator of inflammatory response, including cell proliferation, differentiation and apoptosis [23], it is not surprising that this cytokine can serve as a marker for abnormal response in subjects with ASD. On the other hand, IL-1RA binds to the cell surface IL-1 receptor, inhibits the activities of IL-1β, and modulates IL-1-

Table 1. Demographic and clinical characteristics.

Characteristic	Mean (SD) [Range]	
	Control, *N*=28	ASD, *N*=28
Age, years	12.3 (2.3) [7–15]	12.1 (3.3) [7–15]
Full IQ	101.5 (11.5) [82–124]	99.6 (18.6) [72–136]
Height, cm	149.6 (12.5) [121.4–172.8]	147.1 (17.0) [110.0–175.0]
Weight, kg	40.4 (10.6) [24.0–62.0]	40.4 (13.2) [17.5–72.4]
BMI, kg/m^2	17.7 (2.4) [14.4–25.3]	18.1 (2.6) [13.9–24.2]
Scores on Autism Diagnostic Interview-Revised		
Domain A (social)	N/A	19.9 (5.2) [9–27]
Domain BV (communication)	N/A	13.7 (4.0) [8–21]
Domain C (stereotype)	N/A	5.6 (2.2) [3–10]
Domain D (age of onset)	N/A	3.2 (1.1) [1–5]

Abbreviations: ASD, autism spectrum disorder; IQ, intelligence quotient; BMI, body-mass index; and N/A, not applicable.

doi:10.1371/journal.pone.0020470.t001

Table 2. List of analytes in the multiplex assay.

	Control group (n=28)		ASD group (n=28)			FDR- corrected *P* value
Analytes	mean	SD	mean	SD	*t* value	
Group I						
IL-1β	1.1	0.8	1.7	0.8	−2.616	*0.049
IL-1RA	85.4	49.2	135.0	43.4	−4.002	*0.003
IL-2	BDR		BDR		-	-
IL-4	2.1	0.9	2.7	0.9	−2.487	0.06
IL-5	2.8	1.4	3.8	1.3	−2.906	*0.033
IL-6	5.9	3.2	6.8	2.4	−1.207	0.37
IL-7	10.8	3.1	12.8	3.4	−2.201	0.09
IL-8	8.7	3.7	11.6	2.5	−3.391	*0.014
IL-9	13.0	10.1	14.8	9.9	−0.672	0.68
IL-10	2.5	1.8	2.9	1.4	−1.039	0.45
IL-12 (p70)	21.3	12.6	38.1	13.7	−4.784	*0.001
IL-13	11.8	5.2	16.3	5.0	−3.329	*0.011
IL-15	BDR		BDR		-	-
IL-17	7.2	4.8	17.7	11.9	−4.287	*0.002
Eotaxin	86.7	50.8	107.6	35.3	−1.789	0.16
Basic FGF	BDR		BDR		-	-
G-CSF	4.8	3.4	6.9	3.2	−2.368	0.07
GM-CSF	BDR		BDR		-	-
IFN-γ	80.0	46.4	107.2	49.6	−2.123	0.10
IP-10	1912.2	3202.5	1075.1	322.0	1.376	0.33
MCP-1	26.4	17.3	28.2	13.9	−0.419	0.83
MIP-1α	6.5	2.6	6.7	2.8	−0.227	0.91
MIP-1β	125.0	45.3	159.8	57.1	−2.527	0.06
PDGF-BB	11053.2	3023.3	12465.3	1548.4	−2.200	0.09
RANTES	6303.5	809.6	6103.5	598.4	1.051	0.46
TNF-α	8.6	9.1	18.0	19.6	−2.316	0.08
VEGF	74.3	65.6	124.9	75.4	−2.682	0.05
Group II						
CTACK	555.8	138.7	606.1	145.8	−1.324	0.33
GRO-α	60.5	38.3	99.0	47.4	−3.347	*0.013
HGF	213.1	83.5	266.0	66.9	−2.619	0.06
IFN-α2	38.4	11.1	38.1	8.2	0.150	0.92
IL-1α	0.5	0.4	0.6	0.4	−0.990	0.47
IL-2Rα	59.6	20.0	56.3	22.2	0.579	0.76
IL-3	17.1	16.6	17.3	9.9	−0.051	0.96
IL-12 (p40)	43.4	23.2	56.2	26.8	−1.907	0.15
IL-16	210.8	90.0	220.6	73.1	−0.447	0.83
IL-18	60.3	24.3	61.3	17.2	−0.171	0.93
LIF	BDR		BDR		-	-
MCP-3	7.2	3.4	5.8	3.7	1.443	0.30
M-CSF	10.7	7.2	13.2	7.6	−1.259	0.35
MIF	78.3	31.3	81.0	28.1	−0.333	0.88
MIG	415.2	270.8	471.3	520.5	−0.505	0.80
β-NGF	3.1	1.9	3.1	1.0	0.059	0.98
SCF	144.0	24.3	150.3	39.3	−0.718	0.68
SCGF-β	29762.4	6331.6	32684.0	5613.8	−1.827	0.16
SDF-1α	162.7	55.7	180.8	43.6	−1.347	0.33

Table 2. Cont.

Analytes	Control group (n = 28) mean	SD	ASD group (n = 28) mean	SD	*t* value	FDR-corrected *P* value
TNF-β	3.4	4.6	3.1	3.4	0.285	0.88
TRAIL	160.7	58.9	133.9	48.9	1.851	0.16

Concentrations of analytes are shown in (pg/ml). Note the statistically significant difference between the two groups (*$P<0.05$ after FDR correction for multiple comparisons). Abbreviations: ASD, autism spectrum disorder; BDR, below the detection range; FDR, false discovery rate; and SD, standard deviation.
doi:10.1371/journal.pone.0020470.t002

related immune responses [23]. In this study, plasma levels of IL-1β in subjects with ASD were significantly and positively correlated with those of IL-1RA, suggesting that IL-1RA might have increased as a negative feedback regulator in response to the elevation of IL-1β levels in ASD.

Increases in plasma levels of IL-5, IL-13 and IL-12p70 in ASD

IL-5 is mainly produced by T helper 2 (Th2) cells and mast cells, and belongs to the Th2 cytokine family [25]. IL-5 stimulates B cells to secrete immunoglobulins and is also a mediator of eosinophil differentiation and activation. Previous studies have reported eosinophilia in children with autism [26,27], though a negative result has also been reported [28]. IL-13 is another Th2 cytokine that stimulates B cells to secrete IgE, which is an important mediator of allergic inflammation. A trend toward elevation in plasma levels of IL-4, the major Th2 cytokine, was also observed in the current study ($t = -2.49$, corrected $P = 0.06$, see Table 2). In contrast, plasma levels of IFN-γ and IL-2, which are Th1 cytokines, were similar between subjects with ASD and controls. Plasma levels of IL-2 and IFN-γ have been reported to be increased in autism ($n = 20$, mean age = 10.7 years) [17]. However, since none of the other studies found elevations of IL-2 or IFN-γ in peripheral samples [9,11,14,18], it was suggested that elevation of these Th1 cytokines may not be common in subjects with ASD. Our findings of increased levels of Th2 cytokines without corresponding changes in Th1 cytokines were consistent with previous *in vitro* studies which demonstrated Th2-preferred responses after stimulation in peripheral blood monocytes from subjects with ASD [26,29]. Since Th2 cells have been shown to play a role in the pathogenesis of allergy [30], our current findings are not inconsistent with the fact that allergy is a common clinical problem in individuals with ASD [31,32].

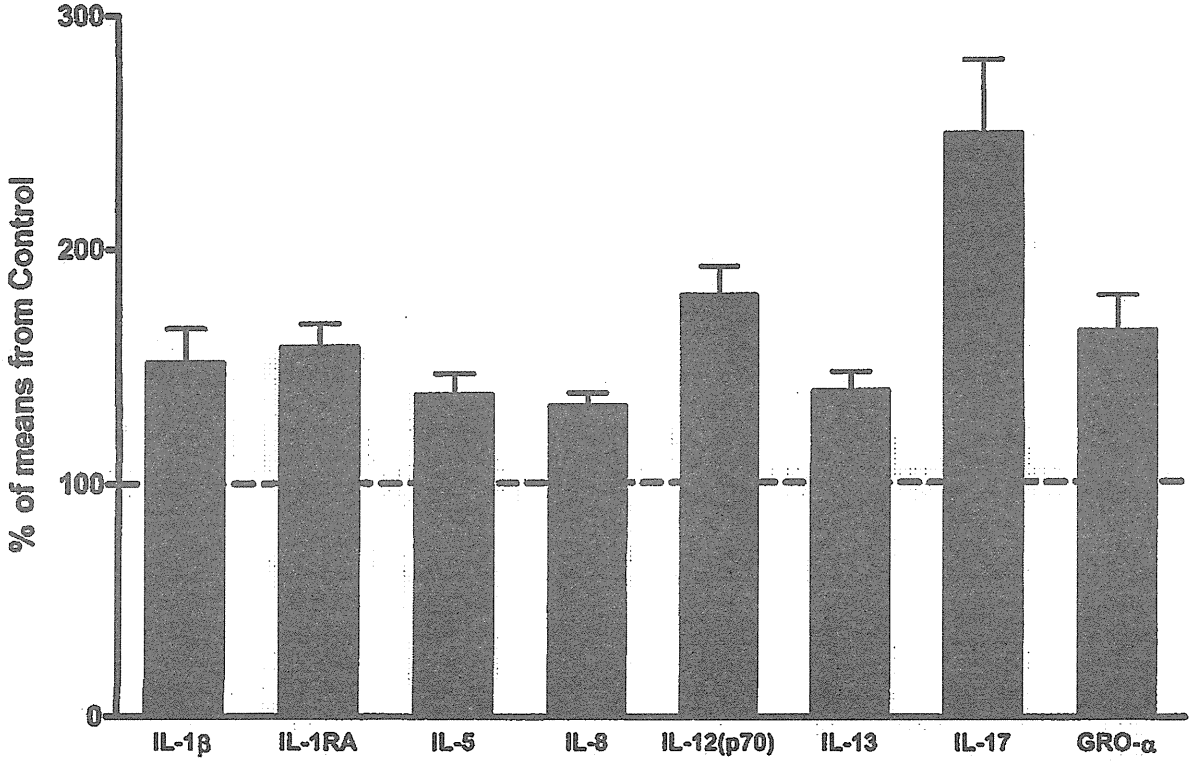

Figure 1. Fold changes of analytes measured by multiplex assay kits in subjects with autism spectrum disorder. The results represent the % concentration relative to the mean concentration of each analyte in the control group (dashed line in red). Data are expressed as the mean plus standard error of the mean.
doi:10.1371/journal.pone.0020470.g001

IL-12(p70) is a heterodimeric cytokine that consists of two subunits, p35 and p40 [33]. Our current finding of elevated plasma levels of IL-12(p70) is consistent with previous results in children with autism [17] and adults with Asperger's syndrome [11]. IL-12(p40) has also been shown to be increased in the plasma of children with ASD [14]. IL-12(p70) is an immunoregulatory cytokine that is produced mainly by B cells and by monocytes, and is involved in the differentiation of naive T cells into Th1 cells [33]. The elevated levels of plasma IL-12 in ASD might be an unsuccessful compensation for the above-mentioned Th1/Th2 imbalance in subjects with ASD.

Increased plasma levels of IL-17, IL-8 and GRO-α in ASD

Outside the Th1/Th2 cell paradigm, a distinct T helper cell subset that produces IL-17 has recently been discovered, and is known as the Th17 cell subset [34]. Th17 cells are responsive to IL23 and secrete IL-17. Enstrom and his colleagues [15] have reported that plasma IL-17 levels in 2- to 5-year-old children with autism were similar to those in controls, but the IL-23 levels were decreased in the children with autism. Researchers from the same group also examined peripheral blood monocytes from subjects with ASD and found that PHA-stimulated release of IL-23, but not IL-17, was lower in subjects with ASD than in controls [35]. The discrepancy between our study and the previous report by Enstrom et al. [15] presumably reflects the differences in the age of participants and experimental designs. In addition, because the kits we used for multiplex assay did not include IL-23 as an analyte, further studies will be needed to examine the effects of this factor. Interestingly enough, however, recent findings suggest that IL-1β, the plasma levels of which were increased in our subjects with ASD, plays an important role in Th17 cell differentiation and IL-17 secretion [36,37].

Both IL-8 and GRO-α are chemokines produced by macrophages and other cell types, such as epithelial and endothelial cells. These chemokines have chemotactic activity on neutrophils and play important roles in the innate immune response. Previous studies have examined IL-8 levels in plasma or serum, and found either increases [14] or no change [11,38] in peripheral IL-8 levels. With regard to GRO-α, there has been no report showing a significant difference as compared to controls. The reason why these chemokines are increased in subjects with ASD is currently unknown. However, IL-17 is known to be a potent mediator of production of IL-8 and GRO-α from epithelial cells [34]. Since IL-8 and GRO-α function as chemotaxins of these chemokines, its elevation in the peripheral circulation suggests an activation of innate immunity. That is, the elevation in plasma IL-8 and GRO-α might have resulted from IL-17 secretion by Th17 cells activated in response to subclinical infections in epithelial or endothelial cells in our subjects with ASD.

Limitations

There were limitations in the present study. The small sample size renders the data presented here preliminary, and a larger study with more subjects with ASD will be necessary. However, recruitment for the current study was limited to a group of high-functioning subjects with ASD, none of whom were given psychotropic drugs. Therefore, our data are free from possible confounding factors and thus reflect a certain common immunological pathology among people with ASD.

Materials and Methods

Subjects

Twenty-eight male subjects with ASD and 28 healthy male controls participated in this study. All the participants were Japanese, born and living in restricted areas of central Japan, including Aichi, Gifu, and Shizuoka prefectures. Based on interviews and available information, including hospital records, diagnoses of ASD were made by an experienced child psychiatrist (TS) based on the DSM-IV-TR criteria [1]. The ADI-R [22] was also conducted by two of the authors (KJT and KM), both of whom have an established reliability of diagnosing autism with the Japanese version of ADI-R. ADI-R is a semi-structured interview conducted with a parent, usually the mother, and is used to confirm the diagnosis and also to evaluate the core symptoms of ASD. The ADI-R domain A score quantifies impairment in social interaction, the domain BV score quantifies impairment in communication, and the domain C score quantifies restricted, repetitive and stereotyped patterns of behavior and interests. The ADI-R domain D corresponds to the age of onset criterion for autistic disorder. We also used the third edition of the Wechsler Intelligence Scale for Children [39] to evaluate the intelligence quotient (IQ) of all the participants. Co-morbid psychiatric illnesses were excluded by means of the Structured Clinical Interview for DSM-IV (SCID) [40]. Participants were excluded from the study if they had any symptoms of inflammation, a diagnosis of fragile X syndrome, epileptic seizures, obsessive-compulsive disorder, affective disorders, IQ of lower than 70, or any additional psychiatric or neurological diagnoses. None of the participants had ever received psychoactive medications before this study. Healthy control subjects were recruited locally by advertisement. All control subjects underwent a comprehensive assessment of their medical history to eliminate individuals with any neurological or other medical disorders. SCID was also conducted to scrutinize any personal or family history of past or present mental illness. None of the comparison subjects initially recruited was found to fulfill any of these exclusion criteria. This study was approved by the ethics committee of the Hamamatsu University School of Medicine. All participants as well as their guardians were given a complete description of the study, and provided written informed consent before enrollment.

Blood sampling and multiplex assay

Fasting blood samples from all the participants were obtained between 11:00 and noon by venipuncture and collected into EDTA-containing tubes. Immediately after the sampling, samples were centrifuged for 10 min at 4°C, divided into 200-μl of aliquots, and stored at −80°C until use. The mean time interval for preparation of plasma from blood samples was 4.5 min (3 to 6 min). Multiplex kits for measuring cytokines and chemokines were purchased from Bio-Rad (Bio-Plex Pro Human Cytokine Group I [27-plex] and Group II [21-plex] panels; Bio-Rad, Hercules, CA). The kits were used per the manufacturer's instructions. Plasma samples were diluted using the appropriate sample diluents provided in each kit in accordance with the manufacturer's instructions. Concentrations (pg/ml) of different analytes in the plasma samples were determined by using the standard curves generated in the multiplex assays. Each standard curve was generated using eight points of concentrations, and a nonlinear least squares minimization algorithm was used for the curve fitting by the five-parameter logistic equation and to determine the high and low limits of detection. Data points for analytes that were occasionally above or below the detection range were discarded.

Data analysis

Comparisons of concentrations of analytes between subjects with ASD and controls were made by an unpaired *t*-test after confirming that there were no statistically significant differences in

variance as assessed by the F test. A P value of less than 0.05 was considered to be statistically significant after adjustment for the false discovery rate (FDR) for multiple comparisons using the Benjamin-Hochberg procedure. Evaluation of relationships between plasma levels of analytes and clinical variables among subjects with autism spectrum disorder was performed with Pearson's r correlation coefficient. In the correlation analysis, values of $P<0.05$ were regarded as statistically significant. All statistical analyses were performed using SPSS statistics software (version 17; SPSS K.K., Tokyo, Japan).

References

1. American Psychiatric Association (2000) Diagnostic and statistical manual of mental disorders. Fourth edition, text revision. Washington, DC: American Psychiatric Association.
2. Lichtenstein P, Carlström E, Råstam M, Gillberg C, Anckarsäter H (2010) The genetics of autism spectrum disorders and related neuropsychiatric disorders in childhood. Am J Psychiatry 167: 1357–1363.
3. Ashwood P, Wills S, Van de Water J (2006) The immune response in autism: a new frontier for autism research. J Leukoc Biol 80: 1–15.
4. Zimmerman AW (2006) The Immune System. In: The Neurobiology of Autism, second edition. Bauman ML, Kemper TL, eds. Baltimore, ML: The Johns Hopkins University Press.
5. Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, et al. (2009) Elevated immune response in the brain of autistic patients. J Neuroimmunol 207: 111–116.
6. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 57: 67–81.
7. Jyonouchi H, Sun S, Le H (2001) Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autismspectrum disorders and developmental regression. J Neuroimmunol 120: 170–179.
8. Corbett BA, Kantor AB, Schulman H, Walker WL, Lit L, et al. (2007) A proteomic study of serum from children with autism showing differential expression of apolipoproteins and complement proteins. Mol Psychiatry 12: 292–306.
9. Croonenberghs J, Bosmans E, Deboutte D, Kenis G, Maes M (2002) Activation of the inflammatory response system in autism. Neuropsychobiology 45: 1–6.
10. Croonenberghs J, Wauters A, Devreese K, Verkerk R, Scharpe S, et al. (2002) Increased serum albumin, gamma globulin, immunoglobulin IgG, and IgG2 and IgG4 in autism. Psychol Med 32: 1457–1463.
11. Schwarz E, Guest PC, Rahmoune H, Wang L, Levin Y, et al. (2010) Sex-specific serum biomarker patterns in adults with Asperger's syndrome. Mol Psychiatry; (Published online on Sep. 28, 2010).
12. Singh VK, Warren RP, Odell JD, Cole P (1991) Changes of soluble interleukin-2, interleukin-2 receptor, T8 antigen, and interleukin-1 in the serum of autistic children. Clin Immunol Immunopathol 61: 448–455.
13. Zimmerman AW, Jyonouchi H, Comi AM, Connors SL, Milstien S, et al. (2005) Cerebrospinal fluid and serum markers of inflammation in autism. Pediatr Neurol 33: 195–201.
14. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah I, et al. (2011) Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. Brain Behav Immun 25: 40–45.
15. Enstrom A, Onore C, Hertz-Picciotto I, Hansen R, Croen L, et al. (2008) Detection of IL-17 and IL-23 in Plasma Samples of Children with Autism. Am J Biochem Biotech 4: 114–120.
16. Grigorenko EL, Han SS, Yrigollen CM, Leng L, Mizue Y, et al. (2008) Macrophage migration inhibitory factor and autism spectrum disorders. Pediatrics 122: e438–e445.
17. Singh VK (1996) Plasma increase of interleukin-12 and interferon-gamma. Pathological significance in autism. J Neuroimmunol 66: 143–145.
18. Sweeten TL, Posey DJ, Shankar S, McDougle CJ (2004) High nitric oxide production in autistic disorder: a possible role for interferon-gamma. Biol Psychiatry 55: 434–437.
19. Di Nisio M, Niers TM, Reitsma PH, Buller HR (2005) Plasma cytokine and P-selectin levels in advanced malignancy: prognostic value and impact of low-molecular weight heparin administration. Cancer 104: 2275–2281.
20. Hart JP, Broadwater G, Rabbani Z, Moeller BJ, Clough R, et al. (2005) Cytokine profiling for prediction of symptomatic radiation-induced lung injury. Int J Radiat Oncol Biol Phys 63: 1448–1454.
21. Fujita-Shimizu A, Suzuki K, Nakamura K, Miyachi T, Matsuzaki H, et al. (2010) Decreased serum levels of adiponectin in subjects with autism. Prog Neuropsychopharmacol Biol Psychiatry 34: 455–458.
22. 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.
23. Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW, et al. (2010) IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog 6: e1000661.
24. Emanuele E, Orsi P, Boso M, Broglia D, Brondino N, et al. (2010) Low-grade endotoxemia in patients with severe autism. Neurosci Lett 471: 162–165.
25. Lloyd CM, Hessel EM (2010) Functions of T cells in asthma: more than just T(H)2 cells. Nat Rev Immunol 10: 838–848.
26. Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, Dienger K, et al. (2006) Elevated cytokine levels in children with autism spectrum disorder. J Neuroimmunol 172: 198–205.
27. Renzoni E, Beltrami V, Sestini P, Pompella A, Menchetti G, et al. (1995) Brief report: allergological evaluation of children with autism. J Autism Dev Disord 25: 327–333.
28. Sweeten TL, Posey DJ, McDougle CJ (2003) High blood monocyte counts and neopterin levels in children with autistic disorder. Am J Psychiatry 160: 1691–1693.
29. Gupta S, Aggarwal S, Rashanravan B, Lee T (1998) Th1- and Th2-like cytokines in CD4+ and CD8+ T cells in autism. J Neuroimmunol 85: 106–109.
30. Nakajima H, Takatsu K (2007) Role of cytokines in allergic airway inflammation. Int Arch Allergy Immunol 142: 265–273.
31. Magalhães ES, Pinto-Mariz F, Bastos-Pinto S, Pontes AT, Prado EA, et al. (2009) Immune allergic response in Asperger syndrome. J Neuroimmunol 216: 108–112.
32. Theoharides TC, Angelidou A, Alysandratos KD, Zhang B, Asadi S, et al. (2010) Mast cell activation and autism. Biochim Biophys Acta (Published online on Dec 28, 2010).
33. Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3: 133–146.
34. Eyerich S, Eyerich K, Cavani A, Schmidt-Weber C (2010) IL-17 and IL-22: siblings, not twins. Trends Immunol 31: 354–361.
35. Onore C, Enstrom A, Krakowiak P, Hertz-Picciotto I, Hansen R, et al. (2009) Decreased cellular IL-23 but not IL-17 production in children with autism spectrum disorders. J Neuroimmunol 216: 126–129.
36. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, et al. (2009) Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity 30: 576–587.
37. Li L, Kim J, Boussiotis VA (2010) IL-1β-mediated signals preferentially drive conversion of regulatory T cells but not conventional T cells into IL-17-producing cells. J Immunol 185: 4148–4153.
38. Nelson PG, Kuddo T, Song EY, Dambrosia JM, Kohler S, et al. (2006) Selected neurotrophins, neuropeptides, and cytokines: developmental trajectory and concentrations in neonatal blood of children with autism or Down syndrome. Int J Dev Neurosci 24: 73–80.
39. Wechsler D (1991) Wechsler Intelligence Scale for Children Third Edition manual. New York, NY: The Psychological Corporation.
40. American Psychiatric Association (1997) User's guide for the structured clinical interview for DSM-IV axis I disorders SCID-I: clinician version. Washington, DC: American Psychiatric Press.

Acknowledgments

The authors thank Mses Tae Takahashi, Erina Sakamoto, and Mika Oyaizu for their excellent technical assistance.

Author Contributions

Conceived and designed the experiments: KS MT TS NM. Performed the experiments: HM KI YK CS. Analyzed the data: KJT YI KN. Wrote the paper: KS NM. Obtained informed consent from participants: SK YY TW KT ST KM.