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Genetic analyses of the brain-derived neurotrophic factor (BDNF) gene in autism

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Abstract

Autism is a severe neurodevelopmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that are detectable in early childhood. Brain-derived neurotrophic factor (BDNF) plays a critical role in the pathogenesis of autism. In this study, we examined the SNP- and haplotypic-association of BDNF with autism in a trios-based association study (the Autism Genetic Resource Exchange). We also examined the expression of BDNF mRNA in the peripheral blood lymphocytes of drug-naïve autism patients and control subjects. In the TDT of autism trios, the SNP haplotype combinations showed significant associations in the autism group. BDNF expression in the drug-naïve autistic group was found to be significantly higher than in the control group. We suggest that BDNF has a possible role in the pathogenesis of autism through its neurotrophic effects on the serotonergic system.

Keywords: Autism; Brain-derived neurotrophic factor; Serotonin transporter; A trios-based association; Peripheral blood lymphocytes

Autism is a severe neurodevelopmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that are detectable in early childhood. The serotonergic system has been found to be developmentally dysregulated in autism; factors that regulate serotonergic neuronal development and serotonin metabolism might have a crucial role in the pathophysiology of autistic disorders caused by the dysfunction of the serotonergic system [1]. Specifically, altered developmental dynamics of serotonin synthesis [2,3] and increases in whole blood serotonin levels have been reported in autistic individuals [4,5]. Effective medications for treating autistic symptoms include drugs that have an impact on the serotonergic system, such as the serotonin receptor antagonists (e.g. Risperidone) and serotonin depleting agents (e.g. Fenfluramine) [6–8].

Multiple lines of evidence suggest that brain-derived neurotrophic factor (BDNF) plays a critical role in the serotonergic function. In the rat brain, BDNF has been found to promote the survival and sprouting of serotonergic axons [9] and the axonal growth of injured serotonergic neurons

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[10,11]. In vitro and in vivo studies support a regulatory role of BDNF in the survival and maturation of serotonergic neurons [12,13]; BDNF has also been shown to modulate serotonergic neurotransmission in vitro [14]. In addition, BDNF administration has been found to increase the synthesis and/or turnover of serotonin in vivo [15–18].

BDNF has a detrimental effect on the aforementioned processes, and has been implicated in the pathogenesis of neurodevelopmental disorders like autism. Specifically, elevated BDNF expression has been observed in the brain [19], blood [20] and serum [21,22] of autistic individuals, compared to healthy controls. Recently, we found that the serum levels of BDNF in patients with autism were significantly lower than those of normal controls [23].

In this study, we examined the SNP- and haplotypic-association of BDNF with autism in a trios-based association study. We also examined the expression of BDNF mRNA in the peripheral blood lymphocytes of drug-naïve autism patients and control subjects, since lymphocytes are now considered to be a convenient and accessible alternative to brain samples for use in biochemical and genetic investigations of the functions of the central nervous system [24].

Materials and methods

Association study

Subjects. The study was approved by the Ethics Committee of the Hamamatsu University School of Medicine.

DNA samples from trios families recruited to the Autism Genetic Resource Exchange (AGRE; http://www.agre.org) were used for this study [25]. We selected trios families, with male offspring scored for autism; additional selection criteria required that (i) there be no possible non-idiopathic autism flag and (ii) all the trios be Whites. Two sets of samples were used in this study; the first set consisted of 104 high-functioning autism (HFA) trios, with the affected offspring having an intelligence quotient (IQ) > 70, whereas, the second set consisted of randomly chosen trios with no IQ information.

Genotyping. The genomic structure of BDNF is based on the UCSC May 2004 draft assembly of the human genome (http://www.genome.ucsc.edu). The BDNF gene is located in 11p14, spanning a genomic stretch of 66.8 kb (mRNA 1580 bases). SNPs were selected based on information from the National Centre for Biotechnology Information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP), The SNP Consortium (TSC: http://www.snp.cshl.org) and the International HapMap Project (http://www.hapmap.org). On the basis of their genomic locations and the minor allele frequencies (MAF) in the Caucasian population, 25 SNPs were chosen for our analysis in order to span the BDNF gene as evenly as possible. Assay-on-demand/Assay-by-design SNP genotyping products (Applied Biosystems, Foster City, CA, USA) were used to score the SNPs based on the TaqMan assay method [26]. Genotypes were determined using the ABI 7900 Sequence Detection System (SDS) (Applied Biosystems) and analyzed using SDS v2.0 software (Applied Biosystems). The SNPs used in the study and their locations are shown in Table 1.

Statistical analysis. PedCheck program v1.1 (http://www.watson.hgen.pitt.edu) was used to identify and eliminate all Mendelian inconsistencies in the trios data set. Markers were tested for association by the conventional transmission disequilibrium test (TDT) using the TDTPHASE program of the UNPHASED software package v2.403 (http://www.hgmp.mrc.ac.uk). All of the three-, four-, and five-marker haplotypes were tested for association in a sliding window across the locus. The option 'drop rare haplotypes' was used to restrict the analysis of haplotypes with a frequency <3%. Pair-wise linkage disequilibrium (LD) between the various markers, based on D' (linkage disequilibrium coefficient) values [27], was estimated using the Haploview software v3.2 (http://www.broad.mit.edu/mpg/haploview); an LD plot was also constructed using this software.

Gene expression analysis

Lymphocyte RNA. The study was approved by the Ethics Committee of the Hamamatsu University School of Medicine. Written informed consent was obtained from each participant after having been provided an explanation of the study procedures. We obtained blood samples from 11 drug-naïve autism patients (age 21.4 ± 2.31 years [mean $\pm$ SD]) and 13 age-matched (22.3 ± 1.93) healthy controls. All the patients and control subjects were males, and were of Japanese origin.

The autism patients were diagnosed according to the Autism Diagnostic Interview-Revised (ADI-R) by trained and certified psychiatrists (K.T.,A.S.) [28]. All of the patients met the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria (American Psychiatric Association, 1994) [29] and International Classification of Diseases, 10th Revision (ICD-10; World Health Organization, 1992) [30], criteria for autism. The patients underwent screening, and were excluded if they had any major medical- or psychiatric-conditions; they had been drug-naïve for at least 6 months. Comorbid anxiety and depressive symptoms were assessed using the Hamilton Anxiety Rating Scale (HAM-A) [31] and the Hamilton Depression rating scale (HAM-D) [32], respectively. Obsessional/repetitive behaviors were clinically rated using the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) [33,34]. Additional aggression symptoms were also assessed using the aggression questionnaire (AQ) [35]. A faux pas detection task was used to measure theory of mind (ToM) [36,37]. All of the evaluations were conducted by a trained research psychiatrist (K.N.).

All of the controls were free of medications, and underwent screening to exclude neurological-, developmental-, or psychiatric-disorders and mental retardation; none of them met any of the relevant criteria of DSM-IV.

Peripheral blood (20 ml) was drawn from the cubital vein into EDTA-containing plastic syringes. Lymphocytes were isolated from blood samples by the Ficoll-Paque gradient method, and total RNA was extracted using RNAzolB reagent (Sawady, Tokyo, Japan) according to the manufacturer's instructions. RNA samples were quantified by analyzing the absorbance at 260 nm in a UV-spectrophotometer. Complementary DNA (cDNA) was synthesized by first strand reverse transcriptase reaction (RT) using Random Primer and M-MLV reverse transcriptase (Invitrogen, CA, USA).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR): Real-time qRT-PCR analysis was performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). TaqMan primer/probes for BDNF and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the endogenous reference, were purchased from Applied Biosystems (Assay-on-Demand™ gene expression products Hs00156058 and Hs99999905, respectively). All reactions were performed in duplicate according to the manufacturer's protocol. A comparative threshold cycle (C_T) method validation experiment was done to check whether the efficiencies of the target and reference amplifications were approximately equal (the slope of the log input amount versus $\Delta C_T < 0.1$). One sample was randomly chosen as a calibrator, and was amplified in each plate to correct for experimental differences among consecutive PCR runs. The amounts of BDNF mRNA were normalized to the endogenous reference, and were expressed relative to the calibrator as $2^{-\Delta\Delta C_T}$ (comparative C_T method).

Statistical analysis. Statistical calculations were performed using SPSS statistical package, version 11.0.1 (SPSS Co. Ltd., Tokyo, Japan) and GraphPad Prism, version 4.00 (GraphPad Software, San Diego, CA, USA). The difference in BDNF expression between the groups was analyzed using the *t*-test. The correlation between various clinical features and BDNF expression was examined using Pearson's correlation coefficient.

Table 1
Single SNP TDT results of BDNF SNPs

Marker	dbSNP ID	Variation[a]	Location (NCBI B34)	Minor allele frequency[b]	HFA trios (IQ > 70)		Random trios	
					T (%)[c]	*p*-value	T (%)[c]	*p*-value
SNP01	rs1491851	A/G	27717072	0.459	47.77	0.4	50.72	0.753
SNP02	rs727155	G/A	27714758	0.044	50.25	0.586	50	1
SNP03	rs1491850	A/G	27714034	0.436	47.66	0.226	50.6	0.74
SNP04	rs908867	G/A	27710073	0.093	49.73	0.75	50.47	0.479
SNP05	rs12273363	A/G	27709168	0.166	50.14	0.893	50.4	0.658
SNP06	rs11030121	G/A	27700516	0.266	49.92	0.961	50.26	0.832
SNP07	rs7934165	C/T	27696292	0.473	52.91	0.239	49.68	0.869
SNP08	rs2030324	T/C	27691224	0.473	52.68	0.281	49.68	0.869
SNP09	rs988748	C/G	27689054	0.264	52.69	0.567	50.18	0.887
SNP10	rs2049046	A/T	27688084	0.458	53.3	0.17	49.29	0.708
SNP11	rs7127507	A/G	27679193	0.28	49.65	0.83	50.59	0.646
SNP12	rs7103411	A/G	27664434	0.264	49.21	0.545	50.03	0.98
SNP13	rs2049045	C/G	27658550	0.226	51.88	0.719	50.18	0.869
SNP14	rs1401635	C/G	27658300	0.268	52.73	0.495	50.61	0.625
SNP15	rs11030104	T/C	27648826	0.256	49.41	0.642	50.37	0.759
SNP16	rs6265 (V66M)	G/A	27644225	0.226	49.7	0.799	49.94	0.957
SNP17	rs7124442	A/G	27641350	0.27	48.97	0.51	50	1
SNP18	rs1519480	A/G	27640021	0.274	49.07	0.554	50.23	0.854
SNP19	rs4923463	T/C	27636809	0.259	49.38	0.624	50.37	0.76
SNP20	rs2203877	A/G	27635219	0.461	53.59	0.141	49.22	0.68
SNP21	rs10501087	A/G	27634417	0.259	49.38	0.624	50.37	0.76
SNP22	rs1519479	G/A	27631840	0.468	54.27	0.092	49.28	0.709
SNP23	rs925946	C/A	27631511	0.263	48.16	0.212	50.37	0.76
SNP24	rs11030096	T/C	27629852	0.461	54.19	0.092	49.29	0.708
SNP25	rs4923461	A/G	27621219	0.256	49.41	0.642	50.14	0.906

HFA, high-functioning autism; T, transmitted.
[a] Common allele is listed first.
[b] Based on the parental genotypes of random trios.
[c] T percentage of common allele is listed.

Results

Association study

Single SNP TDT

TDT was done separately for the HFA trios and for the random trios; the results are shown in Table 1. None of the SNPs showed a significant association in the HFA trios or random trios.

Haplotype TDT

The results of haplotype TDT for HFA- and random trios are shown in Table 2. The three-SNP haplotype combination of SNP04-SNP05-SNP06 ($p = 0.017$), the four-SNP haplotype combination of SNP04-SNP05-SNP06-SNP07 ($p = 0.02$) and the five-SNP haplotype combination of SNP04-SNP05-SNP06-SNP07-SNP08 ($p = 0.02$) showed significant associations in the random group; however, the global values were not significant. None of the three-, four- or five-SNP haplotypes showed significant association in the HFA trios.

LD analysis

LD analysis identified a single haplotype block across the BDNF gene, comprising SNPs 03–25 (Fig. 1).

Lymphocyte gene expression analysis: BDNF expression in the drug-naïve autistic (0.094 ± 0.1 [mean ± SD]) group was found to be significantly higher than in the control (0.034 ± 0.02) group ($t = -2.2$; $df = 22$; $p = 0.039$) (Fig. 2). No significant correlation was observed between any of the clinical features and BDNF expression in the autistic group (Table 3).

Discussion

In the present study, we reported the haplotypic association of BDNF with autism; three-, four-, and five-SNP haplotypes consisting of SNP04 (rs908867), SNP05 (rs12273363) and SNP06 (rs11030121) showed significant associations with random trios. Furthermore, we found that BDNF expression in the drug-naïve autism group was significantly higher than in the control group. To the best of our knowledge, this is the first report demonstrating an association and increased BDNF expression in drug-naïve autism subjects.

The BDNF Val66Met polymorphism (SNP16 in this study) has been reported to be associated with obsessive-compulsive disorder [38], attention deficit hyperactivity disorder [39] and anxiety-related personality traits [40]; this SNP has also been suggested to have a role in the

Table 2
Three-, four- and five-SNP haplotype analysis of BDNF

Three-SNP[a]	*p*-value[b]		Four-SNP[a]	*p*-value[b]		Five- SNP[a]	*p*-value[b]	
	HFA trios	Random trios		HFA trios	Random trios		HFA trios	Random trios
1-2-3	0.151	0.863	1-2-3-4	0.151	0.947	1-2-3-4-5	0.197	0.931
2-3-4	0.658	0.698	2-3-4-5	0.73	0.8	2-3-4-5-6	0.67	0.887
3-4-5	0.499	0.822	3-4-5-6	0.622	0.068	3-4-5-6-7	0.531	0.071
4-5-6	0.698	***0.017***	4-5-6-7	0.576	***0.02***	4-5-6-7-8	0.611	***0.02***
5-6-7	0.721	0.937	5-6-7-8	0.766	0.937	5-6-7-8-9	0.833	0.914
6-7-8	0.636	0.98	6-7-8-9	0.717	0.987	6-7-8-9-10	0.52	0.968
7-8-9	0.635	0.987	7-8-9-10	0.47	0.968	7-8-9-10-11	0.52	0.956
8-9-10	0.49	0.968	8-9-10-11	0.539	0.956	8-9-10-11-12	0.539	0.965
9-10-11	0.634	0.918	9-10-11-12	0.634	0.932	9-10-11-12-13	0.752	0.996
10-11-12	0.634	0.932	10-11-12-13	0.752	0.996	10-11-12-13-14	0.565	0.975
11-12-13	0.702	0.985	11-12-13-14	0.53	0.958	11-12-13-14-15	0.547	0.963
12-13-14	0.538	0.948	12-13-14-15	0.586	0.95	12-13-14-15-16	0.612	0.907
13-14-15	0.681	0.933	13-14-15-16	0.659	0.892	13-14-15-16-17	0.193	0.943
14-15-16	0.686	0.89	14-15-16-17	0.201	0.94	14-15-16-17-18	0.234	0.922
15-16-17	0.555	0.966	15-16-17-18	0.601	0.958	15-16-17-18-19	0.596	0.958
16-17-18	0.582	0.998	16-17-18-19	0.596	0.964	16-17-18-19-20	0.61	0.966
17-18-19	0.43	0.931	17-18-19-20	0.444	0.92	17-18-19-20-21	0.444	0.92
18-19-20	0.4	0.916	18-19-20-21	0.4	0.916	18-19-20-21-22	0.447	0.916
19-20-21	0.352	0.912	19-20-21-22	0.41	0.912	19-20-21-22-23	0.42	0.822
20-21-22	0.41	0.912	20-21-22-23	0.42	0.822	20-21-22-23-24	0.42	0.822
21-22-23	0.32	0.829	21-22-23-24	0.314	0.829	21-22-23-24-25	0.314	0.845
22-23-24	0.253	0.926	22-23-24-25	0.314	0.985			
23-24-25	0.305	0.985						

[a] Based on all possible haplotypes for each combination of SNPs.
[b] Computed on the basis of likelihood ratio test. Significant *p*-values (<0.05) are indicated in bold italics.

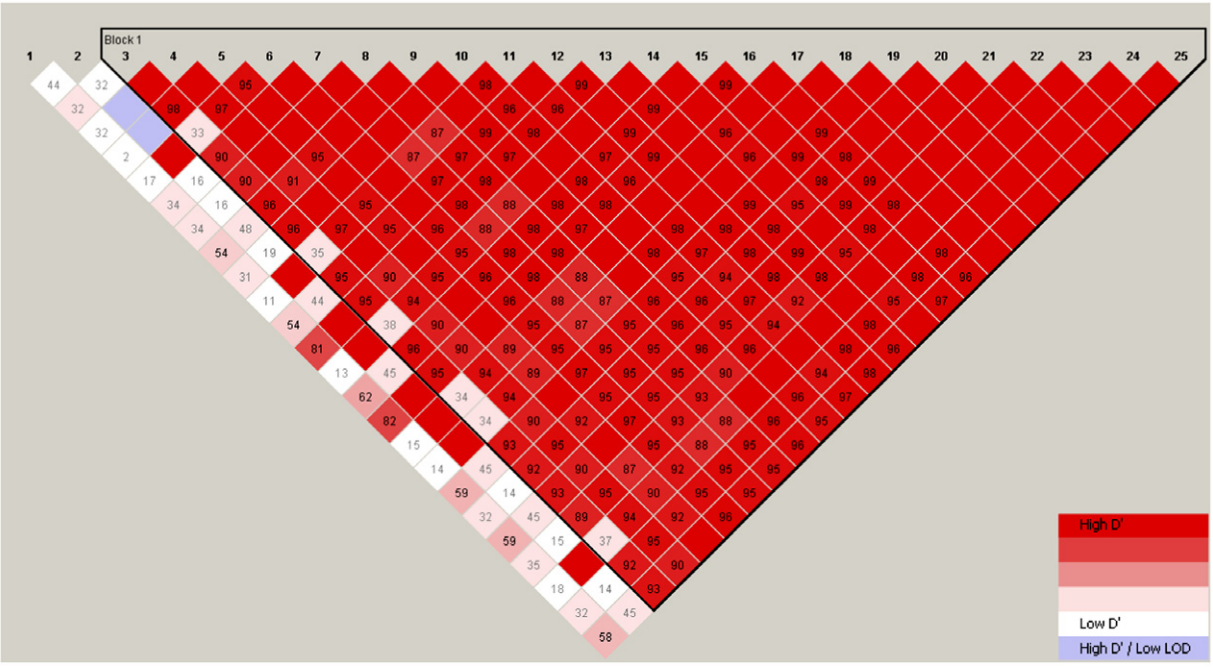

Fig. 1. Haplotype block structure of BDNF based on *D'* values calculated from 148 random trios.

hippocampal and prefrontal cortex functions involved in human memory and learning [41,42]. However, we did not observe any significant association of Val66Met with autism.

Several lines of evidence suggest that BDNF hyperactivity can be deleterious to the neurodevelopmental process, and it has been implicated in the pathophysiology of neurodevelopmental disorders like autism. Specifically, Nelson et al. [20] reported higher BDNF levels in the archived samples of neonatal blood from autistic children compared to normal controls. This finding was further supported by observations of higher concentrations of BDNF in the

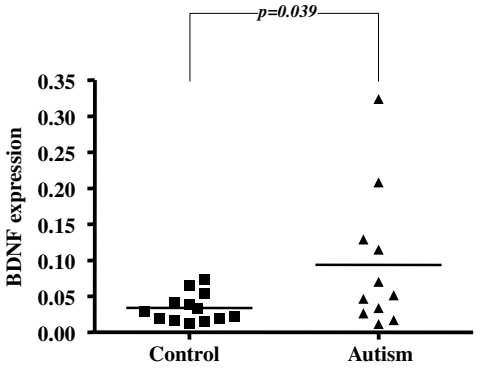

Fig. 2. *t*-test comparison of BDNF mRNA levels in the lymphocytes from control subjects and drug-naïve autism patients. Horizontal bars indicate means. A significant difference in BDNF expression was observed between the two groups ($p = 0.039$).

Table 3
Correlation between clinical features and lymphocyte BDNF expression in the autism group

Clinical feature	Pearson (*r*)	*p*-value
HAM-A	−0.199	0.581
HAM-D	−0.223	0.536
Y-BOCS	−0.141	0.699
Obsession	−0.223	0.536
Compulsion	−0.046	0.899
Aggression questionnaire	0.185	0.610
Faux pas test	0.231	0.530

basal forebrain [19] and in the serum [21,22] of autistic patients compared to healthy controls. In this present study, enhanced BDNF m-RNA expression in the lymphocytes was observed in the drug-naïve autistic group compared to the control group. Therefore, BDNF hyperactivity could result in disruption of the normal developmental program in the brain, leading to abnormalities like overgrowth of brain- and neuronal-tissues, which has been observed in autistic individuals [43–47].

There was no significant correlation between BDNF expression and any of the clinical features of the autistic group. Hence, it may be suggested that elevated BDNF expression is indicative of the disease state per se, and is not dependent on the clinical features of the disease.

Since BDNF has a proven role in regulating the structural [9–13] and functional aspects [14–18] of serotonergic neurons, its hyperactivity might cause dysfunction of the serotonergic system. In a B lymphoblast model, which had several molecular and functional similarities to serotonergic neurons, BDNF treatment was found to decrease serotonin uptake by serotonin transporters, thereby increasing extracellular serotonin levels [48].

Given the critical role of BDNF in brain development, our findings lead us to the hypothesis that enhanced levels of BDNF may contribute to the pathophysiology of autism. It is therefore of great interest to measure BDNF levels in children with autism in order to determine the role of BDNF as a serological marker in children who will go on to develop an autistic disorder.

In conclusion, we suggest that BDNF hyperactivity may play a role in the pathogenesis of autism through its neurotrophic effects on the serotonergic system. Moreover, this is the first report of a genetic association between BDNF and autism; however, replication of these findings and further studies of the functional impact of BDNF in autism are warranted.

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