TABLE I. (*Continued*)

SNP No.	dbSNP ID	Subjects	n	Genotype count (frequency)			$P_{genotypic}$	Allele count (frequency)		$P_{allelic}$	HWE *P*
19	rs3087879			GG	GC	CC		G	C		
		Sz	574	432 (0.75)	131 (0.23)	11 (0.02)		995 (0.87)	153 (0.13)		0.771
		C	568	422 (0.74)	127 (0.22)	19 (0.03)	0.32	971 (0.85)	165 (0.15)	0.41	0.018

$P_{genotypic}$, the Cochran–Armitage trend test; $P_{allelic}$, Fisher's exact test.
[a]Permutation *P*-value = 0.02.

internal control (*GAPDH*), and the mean of the three replicate measures was assigned to each individual.

Statistical Analysis

Allelic and genotypic associations were evaluated by Fisher's exact test and the Cochran–Armitage trend test, respectively. The detection power with this sample size was greater than 0.95 assuming an allelic relative risk of 1.23 and risk allele frequencies from 0.2 to 0.8 according to the Genetic Power Calculator in the total subjects [Purcell et al., 2003]. Deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the chi-squared test. Linkage disequilibrium and haplotype frequencies/associations were evaluated with the Haploview program (http://www.broad.mit.edu/mpg/haploview/). In this study, we evaluated 19 SNPs for allelic associations with schizophrenia in the screening population, and subsequently genotyped SNPs with $P < 0.05$ at the screening step to confirm the association in the replication population. Corrected *P*-values were calculated with the Bonferroni method for SNP association analysis and with the use of 100,000 permutation as implemented in the Haploview program for haplotype association analysis.

Differences in *SLC1A1* expression as determined by real-time quantitative PCR were analyzed by the Wilcoxon test with JMP software version 8 (SAS Institute, Cary, NC), and $P < 0.05$ was considered significant.

RESULTS

The genotype and allele distributions of the 19 tagSNPs in the screening population are shown in Table I. Four SNPs (rs10814995, rs1980943, rs7022369, and rs4641119) showed nominally significant allelic association with schizophrenia. Among them, the genotype distribution of rs7022369 deviated significantly from the HWE in both patient and control groups (Table I). Because SNP rs7022369 is located in the CNV region (Database of Genomic Variants, http://projects.tcag.ca/variation/ variation_33067, 10284, and 2785, http://projects.tcag.ca/variation/), we determined the boundary of the CNV region (Fig. 1) and developed a method to identify the CNV by PCR. The CNV was deleted between 4516798 and 4526818 (NCBI ref: NT 008413.18; Fig. 1d) with an allele frequency of 2%. The CNV was not significantly associated with schizophrenia (Table II). When individuals with the CNV were excluded, the genotype distribution of rs7022369 did not deviate from HWE in the control subjects (Table II). Therefore, we excluded individuals with the CNV in the following analysis for this SNP. Among four SNPs with nominally significant association in the screening subjects, rs7022369 was associated with schizophrenia in an independent case–control population even after Bonferroni correction (allelic nominal *P*-value = 0.001; allelic corrected *P*-value = 0.004 in the same direction as in the screening subjects; Table II). The genotype distribution of rs7022369 did not deviate significantly from HWE in the replication and total samples when individuals with the CNV were excluded (Table II). The data in the combined populations revealed significant allelic associations of rs7022369 (nominal allelic $P = 5 \times 10^{-5}$, allelic OR = 1.30, 95% CI: 1.14–1.47) and rs4641119 (nominal allelic $P = 5 \times 10^{-4}$, allelic OR = 1.24, 95% CI: 1.10–1.41; Table II). Haplotype analysis with rs7022369 and rs4641119 showed that the haplotype frequency of the C of rs7022369 and A of rs4641119 was significantly higher in the schizophrenia group (0.84) than the control group (0.80; permutation $P = 1.0 \times 10^{-3}$).

Because the SNPs associated with schizophrenia are in the haplotype blocks that include exon 2, we resequenced exon 2 in 32 randomly selected patients. However, we did not identify any nonsynonymous mutations. Therefore, we suspected that the SNPs associated with schizophrenia found in the present study were markers regulating *SLC1A1* expression. We explored the association of rs7022369 and rs4641119 with *SLC1A1* expression in the postmortem prefrontal cortex of 43 individuals with schizophrenia and 11 control subjects. *SLC1A1* expression was higher in brains homozygous for the major C allele of rs7022369 or the major A allele of rs4641119 than brains with the other genotypes ($P = 0.003$ and $P = 0.02$, respectively, Wilcoxon test; Fig. 2). This association was particularly obvious in the patient group ($P = 0.01$ at rs7022369 and $P = 0.12$ at rs4641119, Wilcoxon test). However, we should take into account the fact that the number of control brain samples was small. The effects on gene expression of sample pH, postmortem interval, sex, or age at death were not significant (data not shown). *SLC1A1* expression was not significantly different between the patient and control groups ($P = 0.17$, Wilcoxon test).

DISCUSSION

The present study identified the association between SNPs near exon 2 of the *SLC1A1* gene and schizophrenia. These findings need to be replicated in other populations before accepting them. Because the OR of rs7022369 for association with schizophrenia was only 1.30 (95% CI: 1.14–1.47), more than 1,500 patients and an equal number of controls need to be examined to exceed 80% power in replication studies.

In the present study, we did not provide evidence that the SNPs examined directly cause the association with schizophrenia and/or

TABLE II. Genotypic and Allelic Distributions of the SLC1A1 Gene Polymorphisms in the Replication and Combined Populations

SNP no.	dbSNP ID/population	Subjects	n	Genotype count (frequency)			$P_{genotypic}$	Allele count (frequency)		$P_{allelic}$	Allelic OR (95% CI)	HWE *P*
7	rs10814995			AA	AG	GG		A	G			
	Screening	Sz	572	310 (0.54)	222 (0.39)	40 (0.07)		842 (0.74)	302 (0.26)			0.976
		C	561	278 (0.50)	227 (0.40)	56 (0.10)	0.04	783 (0.70)	339 (0.30)	0.04		0.338
	Replication	Sz	1,324	738 (0.56)	494 (0.37)	92 (0.07)		1970 (0.74)	678 (0.26)			0.453
		C	1,323	680 (0.51)	540 (0.41)	103 (0.08)	0.03	1900 (0.72)	746 (0.28)	0.02		0.769
	Combined	Sz	1,896	1048 (0.55)	716 (0.38)	132 (0.07)		2812 (0.74)	980 (0.26)			0.520
		C	1,884	958 (0.51)	767 (0.41)	159 (0.08)	0.004	2683 (0.71)	1085 (0.29)	0.004	1.16 (1.05–1.28)	0.754
9	rs1980943			AA	AG	GG		A	G			
	Screening	Sz	572	183 (0.32)	292 (0.51)	97 (0.17)		658 (0.58)	486 (0.42)			0.29
		C	571	153 (0.27)	289 (0.51)	129 (0.23)	0.03	595 (0.52)	547 (0.48)	0.01		0.74
	Replication	Sz	1,337	432 (0.32)	638 (0.48)	267 (0.20)		1502 (0.56)	1172 (0.44)			0.26
		C	1,304	389 (0.30)	639 (0.49)	276 (0.21)	0.37	1417 (0.54)	1191 (0.46)	0.09		0.65
	Combined	Sz	1,909	615 (0.32)	930 (0.49)	364 (0.19)		2160 (0.57)	1658 (0.43)			0.71
		C	1,875	542 (0.29)	928 (0.49)	405 (0.22)	0.04	2012 (0.54)	1738 (0.46)	0.01	1.13 (1.03–1.23)	0.83
11	rs7022369			CC	CG	GG		C	G			
	Screening	Sz	551	416 (0.75)	115 (0.21)	20 (0.04)		947 (0.86)	155 (0.14)			0.001
		C	541	364 (0.67)	156 (0.29)	21 (0.04)	0.01	884 (0.82)	198 (0.18)	0.01		0.41
	Replication	Sz	1,275	937 (0.73)	312 (0.24)	26 (0.02)		2186 (0.86)	364 (0.14)			0.996
		C	1,271	870 (0.68)	359 (0.28)	42 (0.03)	0.009	2099 (0.83)	443 (0.17)	**0.001**		0.508
	Combined	Sz	1,826	1353 (0.74)	427 (0.23)	46 (0.03)		3133 (0.86)	519 (0.14)			0.08
		C	1,812	1234 (0.68)	515 (0.28)	63 (0.03)	$\mathbf{6.8 \times 10^{-5}}$	2983 (0.82)	641 (0.18)	$\mathbf{5 \times 10^{-5}}$	1.30 (1.14–1.47)	0.309
	rs7022369			C del	G del	del del						
	Individuals with the CNV	Sz	89	79 (0.89)	6 (0.07)	4 (0.04)						
		C	87	76 (0.87)	8 (0.09)	3 (0.03)						
	CNV			2 Copies	1 Copy	0 Copy		Without CNV	With CNV			
	(Combined population)	Sz	1,915	1826 (0.95)	85 (0.04)	4 (0.00)		3737 (0.98)	93 (0.02)			0.006
		C	1,899	1812 (0.95)	84 (0.04)	3 (0.00)	0.93	3708 (0.98)	90 (0.02)	0.88		0.055
13	rs4641119			AA	AC	CC		A	C			
	Screening	Sz	573	431 (0.75)	128 (0.22)	14 (0.02)		990 (0.86)	156 (0.14)			0.23
		C	576	384 (0.67)	170 (0.30)	22 (0.04)	0.001	938 (0.81)	214 (0.19)	**0.001**		0.56
	Replication	Sz	1,342	983 (0.73)	325 (0.24)	34 (0.03)		2291 (0.85)	393 (0.15)			0.25
		C	1,341	927 (0.69)	382 (0.28)	32 (0.02)	0.02	2236 (0.83)	446 (0.17)	0.02		0.32
	Combined	Sz	1,915	1414 (0.74)	453 (0.24)	48 (0.03)		3281 (0.86)	549 (0.14)			0.11
		C	1,917	1311 (0.68)	552 (0.29)	54 (0.03)	5.9×10^{-4}	3174 (0.83)	660 (0.17)	$\mathbf{5 \times 10^{-4}}$	1.24 (1.10–1.41)	0.65

NV region: chromosome 4516798–4526818 (NCBI ref:NT 008413.18); $P_{genotype}$: Cochran–Armitage trend test.

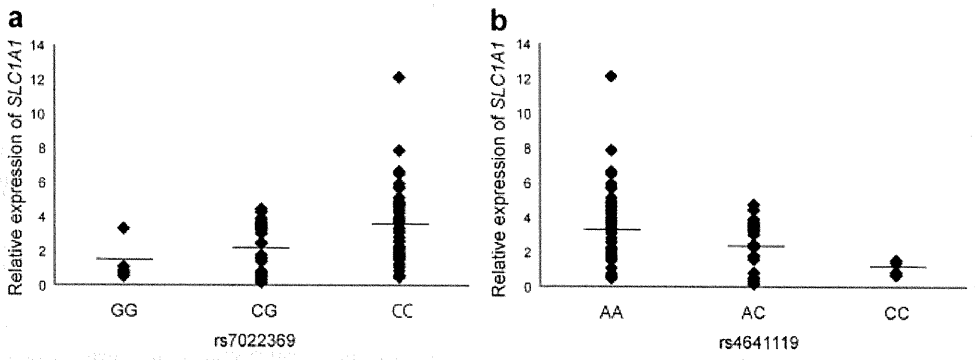

FIG. 2. Expression of *SLC1A1* in postmortem brains classified according to the single nucleotide polymorphism rs10758629 and rs4641119 genotype. Expression of *SLC1A1* was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. a: The difference in expression between the TT genotype and AA genotype in rs10758629 is significant (Wilcoxon test, $P = 0.003$). AA genotype, $n = 7$; TA genotype, $n = 30$; TT genotype, $n = 52$. b: The difference in expression between the AA genotype and CC genotype in rs4641119 is significant (Wilcoxon test, $P = 0.02$). CC genotype, $n = 7$; AC genotype, $n = 28$; AA genotype, $n = 52$. The horizontal line indicates the mean.

the association of *SLC1A1* expression in the prefrontal cortex. A survey of 193 neuropathologically normal human brain samples (Myers et al., 2007) showed the location of a potential *cis*-acting region regulating *SLC1A1* expression within the 15 kb between rs1980943 and rs10758629, as calculated with PLINK [Purcell et al., 2007], where rs7022369 is located. The calculated lowest allelic *P*-value of 0.006 was at rs10814997, which is in complete linkage disequilibrium with rs1980943 (according to the HapMap data, $r^2 = 1$ in the Japanese population). An association between rs1980943 and schizophrenia was suggested in the present study (nominal allelic $P = 0.01$, Table II). Thus, the *cis*-acting region regulating *SLC1A1* is likely to be located in the first intronic region, although its exact position requires further investigation.

Decreases in EAAT3 have been observed in the striatum of schizophrenics [McCullumsmith and Meador-Woodruff, 2002; Nudmamud-Thanoi et al., 2007]. Preclinical studies have demonstrated that chronic treatment with clozapine or haloperidol can downregulate EAAT3 in the infralimbic cortex and hippocampal CA2 [Schmitt et al., 2003]. Therefore, EAAT3 expression is influenced by antipsychotic treatments, but it is difficult to distinguish between the cause and effect on the basis of postmortem brain studies. In the model of diminished glutamate activity in schizophrenia, potential therapeutic effects on some symptom dimensions is expected by glutamate re-uptake inhibitors, such as EAAT3 antagonist, which could increase the synaptic availability of glutamate and increase glutamatergic action at the postsynaptic neuron [Miyamoto et al., 2005]. In the present study, the risk genotype was associated with increased *SLC1A1* expression levels in the prefrontal cortex. On the basis of these findings, we speculated that individuals with a tendency toward increased EAAT3 expression are susceptible to schizophrenia. Higher EAAT3 may be linked to lower synaptic availability of glutamate or more direct mechanism(s) leading to improper functioning of NMDA receptors in some cases. Because different regulation of EAAT3 among brain regions is likely and the associations between SNPs and *SLC1A1* expression were not analyzed in regions other than the prefrontal cortex, further studies regarding the same are required. Furthermore, in our findings, the relationship between SNPs and *SLC1A1* expression in the prefrontal cortex was observed more obviously in the patient group than the control group. Therefore, the possibility remains that the association between SNPs and *SLC1A1* expression reflected antipsychotic treatment responses.

The polymorphisms in *SLC1A1* have been reported to be associated with obsessive-compulsive disorder [Arnold et al., 2006; Dickel et al., 2006; Grados and Wilcox, 2007; Stewart et al., 2007]. More recently, a *SLC1A1* haplotype was reported to be associated with obsessive-compulsive symptoms induced by atypical antipsychotics [Kwon et al., 2009]. These polymorphisms that were associated with obsessive-compulsive disorder or other symptoms span from introns 2 to 6 of the *SLC1A1* gene, and they are not in linkage disequilibrium with SNPs identified as associated with schizophrenia in the present study (Fig. 1).

In conclusion, our findings provide evidence that the *SLC1A1* gene might be involved in susceptibility to schizophrenia. Further studies on the involvement of the *SLC1A1* gene in the pathophysiology of schizophrenia and confirmation of the present association in other populations are necessary.

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ORIGINAL ARTICLE

DPP6 as a candidate gene for neuroleptic-induced tardive dyskinesia

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We implemented a two-step approach to detect potential predictor gene variants for neuroleptic-induced tardive dyskinesia (TD) in schizophrenic subjects. First, we screened associations by using a genome-wide (Illumina HumanHapCNV370) SNP array in 61 Japanese schizophrenia patients with treatment-resistant TD and 61 Japanese schizophrenia patients without TD. Next, we performed a replication analysis in 36 treatment-resistant TD and 138 non-TD subjects. An association of an SNP in the *DPP6* (dipeptidyl peptidase-like protein-6) gene, rs6977820, the most promising association identified by the screen, was significant in the replication sample (allelic $P=0.008$ in the replication sample, allelic $P=4.6\times10^{-6}$, odds ratio 2.32 in the combined sample). The SNP is located in intron-1 of the *DPP6* gene and the risk allele was associated with decreased *DPP6* gene expression in the human postmortem prefrontal cortex. Chronic administration of haloperidol increased *Dpp6* expression in mouse brains. DPP6 is an auxiliary subunit of Kv4 and regulates the properties of Kv4, which regulates the activity of dopaminergic neurons. The findings of this study indicate that an altered response of Kv4/DPP6 to long-term neuroleptic administration is involved in neuroleptic-induced TD. *The Pharmacogenomics Journal* advance online publication, 9 August 2011; doi:10.1038/tpj.2011.36

Keywords: DPP6; dopamine; schizophrenia/antipsychotics; tardive dyskinesia; Kv4

Introduction

Tardive dyskinesia (TD) is the involuntary movement of the tongue, lips, face, trunk and extremities that occurs in patients who are undergoing long-term treatment with antipsychotic medication. TD is often intractable to treatment and the presence of intractable TD is associated with a poorer quality of life.[1] Even though recent studies have indicated that most patients have no significant interference in functioning or quality of life from TD,[2,3] identifying patients at high risk for TD is still a high priority for psychiatrists in treatment selection. Second-generation antipsychotics have lowered the risk of TD to approximately 1% annually as compared with the 5% frequency with typical agents,[4,5] although a recent review has reported a much higher annual TD incidence of 3.9% for second-generation antipsychotics as compared with 5.5% for typical agents.[6] Furthermore, because second-generation antipsychotics may have few other advantages over older, cheaper drugs, doubt has been raised about the cost-effectiveness of second-generation antipsychotics when based purely on this reduced risk of TD.[2] Owing to the lack of effective treatments for TD, its therapeutic management can be problematic for schizophrenia patients receiving antipsychotic medications, especially for those patients who develop severe intractable TD. Therefore, the strategies to prevent TD are often discussed in the context of the safety and use of antipsychotic drugs.[7]

It is not known why only some patients develop TD, that is, the determinants of its onset are still unclear. At present the etiology of TD may be related to the interaction between the exogenous drugs and the endogenous predisposition, but the nature of TD is so far elusive. In addition to age, gender and ethnicity as suggested risk factors for TD, smoking, drinking and use of street drugs may also increase risk.[8] There is some evidence for a genetic component to TD[9] and molecular genetic studies of TD were conducted to identify genes related to TD.[10]

The pathophysiology of TD is not completely understood. In addition to the dopamine super-sensitivity hypothesis of TD,[11] there are many other pathophysiological models proposed, including changes in neurotransmitter signaling systems such as γ-aminobutyric acid,[12] norepinephrine,[13] serotonin[14] and acetylcholine,[15] which are affected by neuroleptics. In addition to a candidate gene approach,[16] two genome-wide association studies (GWASs) based on the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study were published.[17,18] We also reported associations between single-nucleotide polymorphisms (SNPs) on the Illumina Human-1 Genotyping 109K BeadChip and TD in the Japanese sample,[19] in which we selected 63 SNPs with allelic *P*-values <0.002 and located within 10 kb from known genes for subsequent replication analysis, and found three SNPs associated nominally significantly with TD in the replication sample. The allelic *P*-values in the combined sample were 2×10^{-5} for rs2445142 in *HSPG2*; 2×10^{-4} for rs4738269 in *KCNB2* and 6×10^{-4} for rs2061051 in *GBRG3*, respectively. We also reported associations of SNPs in the genes grouped into the γ-aminobutyric acid receptor signaling pathway,[7] through GWAS by using the Illumina Human-1 BeadChip in a Japanese population. In the present study, we searched for further SNPs associated with TD by using the Illumina HumanHapCNV370 BeadChip to complement our previous results using the Human-1 BeadChip.

Materials and methods

Ethical considerations

The ethics committee of each institution approved the study. Written informed consent was obtained from all patients after adequate explanation of the study.

Human subjects

The human subjects in this study were 97 Japanese schizophrenia patients with treatment-resistant TD and 199 Japanese schizophrenia patients without TD (Table 1), most of whom have been described elsewhere.[7] In brief, subjects were identified at psychiatric hospitals located around the Tokyo and Nagoya areas of Japan. All patients fulfilled the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV[20] for schizophrenia. All subjects and their parents were of Japanese descent. All subjects had been receiving antipsychotic therapy for at least 1 year and their TD status was monitored for at least 1 year. TD was assessed according to the Japanese version of the Abnormal Involuntary Movement Scale (AIMS), which was validated by Itoh *et al.* (1977; in Japanese).[21] TD was diagnosed according to the criteria proposed by Schooler and Kane.[22] Once TD was identified, the patients were followed up and received standard therapeutic regimens for TD to minimize TD symptoms. If TD persisted after more than 1 year of therapy, patients were considered potential treatment-resistant TD patients. Treatment-resistant TD patients were defined as those patients with dyskinetic movements that persisted more than 1 year and did not improve after at least 1 year of appropriate treatment following guideline-recommended therapeutic regimens for TD. Patients with treatment-resistant TD were all inpatients who had been receiving antipsychotic therapy for controlling both psychosis and persistent severe TD. The treatment options for TD include possible reduction of antipsychotics, as well as switching from conventional antipsychotics to atypical ones, without relapse of their psychotic conditions. The TD status, as well as psychotic conditions, had been checked every 2 weeks for more than 1 year. Based on these observations, the types and the doses of antipsychotic medications were adjusted and determined. We hypothesized that treatment-resistant TD, a severe form of TD, was suitable for detection of genetic association with TD. Only treatment-resistant TD patients were included as those affected with TD in this study. Patients in whom TD never developed despite antipsychotic therapy for more than 10 years were recruited as control patients.

Genotyping and statistics

Association screening was performed by using the Illumina HumanHapCNV370 Chip according to the manufacturer's

Table 1 Clinical characteristics of patients in the TD group and the non-TD group

	Genome-wide sample		*Confirmation sample*	
	TD (n = 61)	*Non-TD (n = 61)*	*TD (n = 36)*	*Non-TD (n = 138)*
Male:female ratio	35:26	35:26	18:18	88:50
Age (years)	57.3 ± 17.3	58.1 ± 12.3	58.0 ± 15.7	55.5 ± 1.0
Duration of illness (years)	35.6 ± 18.3	33.7 ± 12.5	37.3 ± 14.1	35.3 ± 1.02
Current neuroleptic dose (chlorpromazine-eq; mg year^{-1})	133 132 ± 201 021	469 497 ± 901 846	132 550 ± 86 292	407 456 ± 42 245

Abbreviation: TD, tardive dyskinesia.
The values are the means ± s.d. or number of patients.
Chlorpromazine-eq: chlorpromazine equivalents.

protocol (Illumina, San Diego, CA, USA). All DNA samples were subjected to rigorous quality control to check for fragmentation and amplification. SNPs on autosomal chromosomes ($n=290\,527$) were extracted. Owing to the small sample size and the fact that gender is not known to have a definite effect on TD, we did not analyze SNPs on the X chromosome. No subjects had genotype call rates <97%. The average genotype call rate was 99.7% and the mean heterozygosity of all SNPs was 30%. Two duplicate pairs of samples were genotyped and showed 99.9% genotype identity. SNPs with more than 5% missing genotypes ($n=2853$) and those with minor allele frequency <1% ($n=28\,930$) among subjects were excluded. For missing genotypes <5%, SNPs deviating from Hardy–Weinberg equilibrium ($P<0.0001$; $n=1040$) were excluded. A total of 257 704 autosomal SNPs passed quality control in the sample.

Replication analysis was performed by genotyping SNPs by the TaqMan method. Allelic discrimination was performed by using the ABI PRISM 7900HT Sequence Detection System, by using the SDS 2.0 software (Applied Biosystems, Foster City, CA, USA). Genotyping using TaqMan probes (Applied Biosystems) was performed twice for each SNP, and genotype concordance was 99.7%. Genotyping completeness was >0.99. We treated those uncalled or discrepant genotypes as missing genotypes. Haplotype blocks in the DPP6 (dipeptidyl peptidase-like protein-6) gene were visualized by using the Haploview program (http://www.broad.mit.edu/mpg/haploview/).

Allelic associations between SNPs and TD, and departure from Hardy–Weinberg equilibrium, were evaluated by χ^2-test or Fisher's exact test. Bonferroni's correction for multiple comparisons was applied.

An association was considered significant when the allelic *P*-value was less than 1.9×10^{-7} in the screening step and allelic *P*-value (one-tailed) was <0.05 after Bonferroni's correction for the number of SNPs examined in the replication step. The power of our sample (case = 61 and control = 61) was more than 0.7, with an α of 1.9×10^{-7} assuming a risk allele frequency of 0.3, a disease prevalence of 0.1 and a genotypic relative risk of 4 under the multiplicative model of inheritance, calculated using Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). The replication sample had a power of more than 0.7 assuming two SNPs examined and a genotypic relative risk of 2 under the same model in the screening sample.

Human postmortem brains

Brain specimens were obtained from individuals of European (Australian) and Japanese descent. The Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls. The diagnosis of schizophrenia was made according to the DSM-IV criteria (American Psychiatric Association, 1994) by a psychiatrist and a senior psychologist. The control subjects had no known history of psychiatric illness. Tissue blocks were cut from the gray matter in an area of the prefrontal cortex referred to as Brodmann's area-9 (BA9). Japanese samples of BA9 gray matter from Japanese brain specimens comprised six schizophrenic patients and 11 age- and gender-matched controls. Details of the condition of the postmortem brains have been provided elsewhere.[23,24]

Analysis of DPP6 transcription in human brain tissue

Total RNA was extracted from human brain tissues by using the ISOGEN Reagent (Nippon Gene Co., Tokyo, Japan). The RNA quality was checked by using a Nanodrop ND-1000 spectrophotometer (LMS, Tokyo, Japan) to yield an optical density (OD) 260/280 ratio of 1.8–2 and an OD 260/230 of 1.8 or greater. The expression of the *DPP6* genes was analyzed by using the TaqMan Real-Time PCR system (Applied Biosystems). From RNA, cDNA was synthesized by using ReverTra Ace (Toyobo, Tokyo, Japan) and oligo-dT primers. The expression of the *DPP6* gene was analyzed by using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with TaqMan gene expression assays for *DPP6* (Hs00157265_m1) and normalized to the expression of Human GAPDH Control Reagents (Applied Biosystems).

The genotype effects on *DPP6* expression were analyzed by analysis of variance followed by *post-hoc* Student's *t*-tests by using JMP software version 7.0.1 (SAS Institute, Cary, NC, USA).

Animals

To examine the effects of long-term antipsychotic treatments on gene expression, we set up two experimental groups. In the treatment group, 4-week-old C57BL/6J male mice were treated with an intraperitoneal injection of $1.0\,mg\,kg^{-1}$ haloperidol ($n=10$) once each day for 50 weeks. The control group was administered vehicle saline ($n=10$) under the same regime. The mice were killed 4 h after the last injection to obtain brain tissues. The prefrontal cortex, midbrain, hippocampus, thalamus and striatum were removed by dissection and total RNA was extracted by using an RNeasy kit (Qiagen K.K., Tokyo, Japan). After cDNA synthesis from total RNA samples, the transcription level of cDNA samples was analyzed by TaqMan Expression assay for Dpp6 (Mm00456605_ml; Applied Biosystems) and normalized to that of rodent *Gapdh* by using Rodent *Gapdh* Control Reagents (Applied Biosystems). The average relative expression levels in the haloperidol-treated group were compared with the saline groups in each region by analysis of variance.

Results

We tested for allelic association between each SNP and TD by using the χ^2-test. The distribution of allelic *P*-values for association of SNPs with TD is shown in Figure 1a along with Figure 1b showing the quantile–quantile plot. The genomic inflation factor was 1.008. We did not find SNPs at the genome-wide significance level ($P<1.9\times10^{-7}$) in the screening sample. Table 2 shows the top 10 SNPs that had an allelic association with TD. The distribution of the genotypes of the 10 SNPs did not deviate from Hardy–Weinberg equilibrium in these SNPs. Three of them were

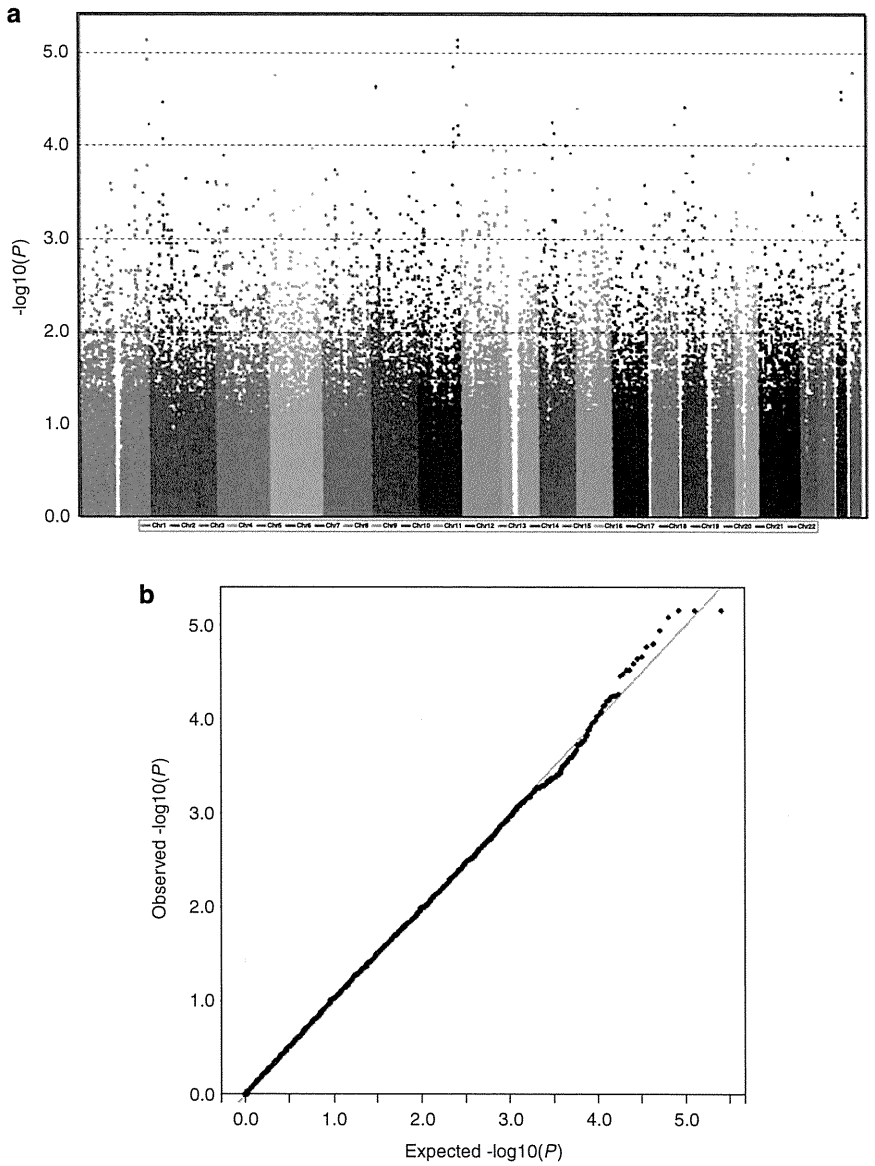

Figure 1 Genome-wide association of SNPs with neuroleptic induced TD. (**a**) The $-\log_{10}$ of uncorrected *P*-values for the association of each SNP with TD is plotted according to its physical position on successive chromosomes. (**b**) The quantile–quantile plot of the observed versus the expected cumulative probabilities for allelic association with TD. SNP, single-nucleotide polymorphism; TD, tardive dyskinesia.

located in the DPP6 gene and two of them were in the *SMYD3* gene. The three SNPs in the *DPP6* gene were in one linkage disequilibrium (LD) block and the two SNPs in the *SMYD3* gene were also in one LD block. Therefore, we selected rs6977820 in the *DPP6* and rs2485914 in the *SMYD3* genes, which showed the most significant *P*-values for TD in each LD block, to replicate the association in an independent population. The association between rs6977820 and TD was significant in the replication sample (allelic *P*-value (one-tailed) = 0.008); however, the association between rs2485914 and TD was not significant (allelic *P*-value (one-tailed) = 0.38) (Table 3). The allelic association *P*-value and odds ratio (95% confidence intervals) between rs6977820 and TD were 4.6×10^{-6} and 2.32 (1.61–3.34) in the combined sample. The distribution of *P*-values in the DPP6 gene in the screening samples is shown in Figure 2.

The SNP rs6977820 was located within intron-1 and the LD block did not extend to the exons. Therefore, we did not re-sequence the exons of the *DPP6* gene. We speculated that the SNP may be associated with the expression levels of *DPP6* and, therefore, we conducted real-time PCR for the association between the rs6977820 and the *DPP6* expression levels in the human postmortem prefrontal cortex. Analysis of variance revealed a significant main effect of genotype ($F(2, 33) = 8.1$, $P = 0.001$). There was no significant effect of population (Australian or Japanese) ($F(1, 35) = 2.2$, $P = 0.15$) or diagnosis (schizophrenia or control) ($F(1, 35) = 1.7$,

Table 2 Top 10 loci ranked by SNP χ^2-test for association with neuroleptic-induced TD in the screening population

Ranking	*SNP (allele)*	*Chr.*	*Position*	*Closest gene*	P-*value*	*Risk allele*	*Risk allele frequency*	
							With TD	*Without TD*
1	rs6977820 (A/G)	7	153 702 953	*DPP6*	7.06×10^{-6}	A	0.43	0.16
2	rs4726411 (A/C)	7	153 705 030	*DPP6*	7.06×10^{-6}	A	0.43	0.16
3	rs2485914 (T/C)	1	244 287 369	*SMYD3*	7.08×10^{-6}	T	0.76	0.48
4	rs1292312 (A/C)	7	153 721 089	*DPP6*	8.33×10^{-6}	A	0.43	0.17
5	rs7523878 (A/G)	1	244 360 339	*SMYD3*	1.14×10^{-5}	G	0.77	0.50
6	rs2833907 (A/G)	21	32 869 651	*TCP10L*	3.02×10^{-5}	G	0.96	0.78
7	rs6705484 (T/G)	2	53 842 748	*ASB3*	3.32×10^{-5}	T	0.26	0.07
8	rs6986075 (A/G)	8	27 230 817	*PTK2B*	3.52×10^{-5}	A	0.89	0.66
9	rs10825371 (A/G)	10	56 105 385	*PCDH15*	5.47×10^{-5}	G	0.72	0.47
10	rs7804017 (T/C)	7	153 710 568	*DPP6*	5.87×10^{-5}	T	0.25	0.07

Abbreviations: SNP, single-nucleotide polymorphism; TD, tardive dyskinesia.

Table 3 Results for two SNPs for association with neuroleptic-induced TD in the screening and replication populations

rs6977820 (DPP6)

Population	*Genotype count (frequency)*			P	*Allele count (frequency)*		P[a]
	GG	*GA*	*AA*		*G*	*A*	
Screening							
With TD ($n=61$)	20 (0.33)	30 (0.49)	11 (0.18)	0.00006	70 (0.57)	52 (0.43)	0.000007
Without TD ($n=61$)	44 (0.72)	14 (0.23)	3 (0.05)		102 (0.84)	20 (0.16)	
Replication							
With TD ($n=36$)	12 (0.33)	16 (0.44)	8 (0.22)	0.04	40 (0.56)	32 (0.44)	0.008
Without TD ($n=137$)	71 (0.52)	54 (0.39)	12 (0.09)		196 (0.72)	78 (0.28)	
Total							
With TD ($n=97$)	32 (0.33)	46 (0.47)	19 (0.20)	0.00007	110 (0.57)	84 (0.43)	0.0000046
Without TD ($n=198$)	115 (0.58)	68 (0.34)	15 (0.08)		298 (0.75)	98 (0.25)	

rs2485914 (SMYD3)

Population	*Genotype count (frequency)*			P	*Allele count (frequency)*		P
	CC	*CT*	*TT*		*C*	*T*	
Screening							
With TD ($n=61$)	5 (0.08)	19 (0.31)	37 (0.61)	0.0001	29 (0.24)	93 (0.76)	0.000007
Without TD ($N=61$)	17 (0.28)	29 (0.48)	15 (0.25)		63 (0.52)	59 (0.48)	
Replication							
With TD ($n=36$)	5 (0.14)	19 (0.53)	12 (0.33)	0.54	29 (0.40)	43 (0.60)	0.38
Without TD ($N=138$)	29 (0.21)	61 (0.44)	48 (0.35)		119 (0.43)	157 (0.57)	
Total							
With TD ($n=97$)	10 (0.10)	38 (0.39)	49 (0.51)	0.002	58 (0.30)	136 (0.70)	0.0002
Without TD ($n=199$)	46 (0.23)	90 (0.45)	63 (0.32)		182 (0.46)	216 (0.54)	

Abbreviations: DPP6, dipeptidyl peptidase-like protein-6; SNP, single-nucleotide polymorphism; TD, tardive dyskinesia.
[a]*P*-value (two-tailed) for the screening and combined sample, and *P*-value (one-tailed) for the replication sample.

$P=0.20$). *Post-hoc* analysis demonstrated that the *DPP6* levels were significantly lower in the AA genotype than in the GG genotype ($P=0.0004$) or in the AG genotype ($P=0.01$). *DPP6* levels were highest in subjects with the GG genotype, lowest in the AA genotype and intermediate in those with the AG genotype (Figure 3).

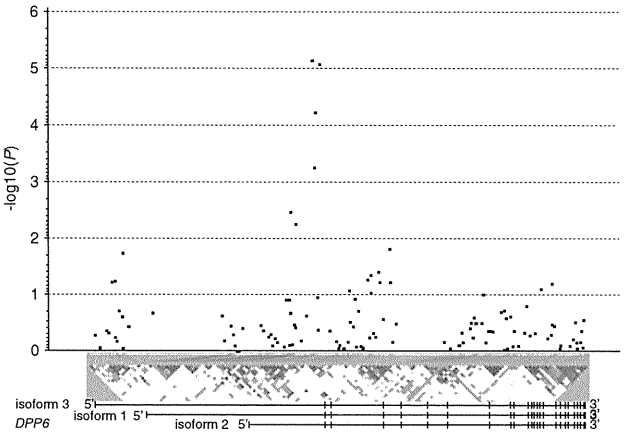

Figure 2 Association of SNPs in the *DPP6* gene with TD in the screening samples. LD in the HapMap data is also shown, with red (black) indicating high LD ($D'>0/8$) and white indicating low LD ($D'<0.7$). Exons are shown in the bottom. *DPP6*, dipeptidyl peptidase-like protein-6; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism; TD, tardive dyskinesia.

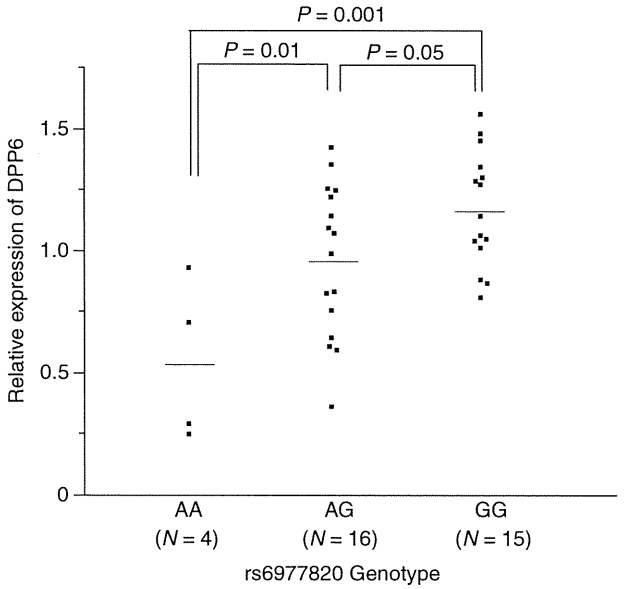

Figure 3 *DPP6* expression levels in the postmortem prefrontal region by genotype. The vertical scores show the average (s.e.m.) relative expression in each of the three genotype groups, compared with the mean gene expression level in the total samples (*P*-values; Student's *t*-test). *DPP6*, dipeptidyl peptidase-like protein-6.

Because TD is caused by long-term use of neuroleptics, we evaluated the effects of long-term administration of haloperidol on the expression of the *Dpp6* gene. Significantly higher expression levels of *Dpp6* were observed in the prefrontal ($F(1, 17)=4.5$, $P=0.05$), striatal ($F(1, 17)=6.7$, $P=0.02$), hippocampal ($F(1, 17)=7.7$, $P=0.01$) and ventricular midbrain ($F(1, 17)=7.9$, $P=0.01$) regions of mice after a 50-week treatment with haloperidol than after a 50-week

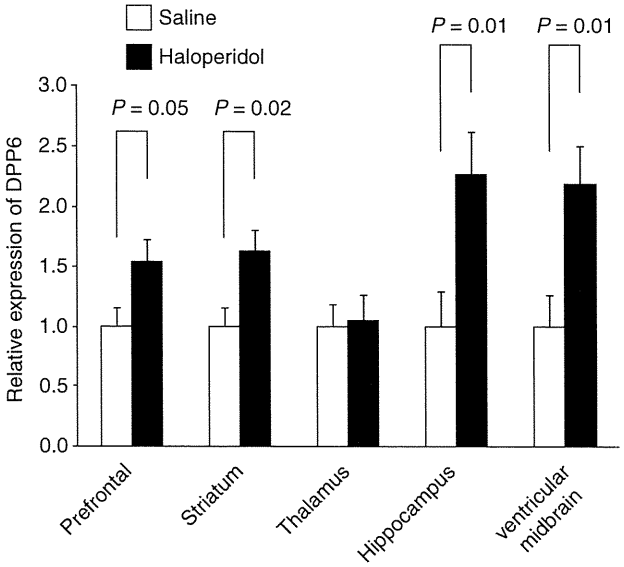

Figure 4 Effect of haloperidol on *Dpp6* gene expression in mouse brains. The relative expression levels of *Dpp6* from the prefrontal cortex, midbrain, hippocampus, thalamus and striatum in mouse brains after treatment with haloperidol for 50 weeks ($n=10$) were compared with those of the saline control group ($n=10$) by using Student's *t*-test. *Dpp6*, dipeptidyl peptidase-like protein-6.

treatment with saline (Figure 4). We did not observe vacuous chewing movements in mice treated with haloperidol during this study.

Discussion

The present study identified an allele or risk genotype in the DPP6 gene, which was associated with TD and lower *DPP6* expression levels in the prefrontal cortex brain. Long-term administration of haloperidol increased the *Dpp6* gene expression in mice. Based on these findings, we hypothesized that long-term administration of neuroleptics increased *DPP6* levels in the brain, and that a genetically based reduction in the ability to respond in this way increases the risk for TD.

There have been no reports on the relationship between DPP6 and movement disorders. The deletion at the *DPP6* locus has been reported in amyotrophic lateral sclerosis and autism.[25,26] The TD-associated SNP found in this study, rs6977820, is not included in the Affymetrix 500K chip. However, rs4726411, which is in LD with rs6977820 ($r^2=0.96$), is included in the Affymetrix 500K chip (http://www.broadinstitute.org/mpg/snap/ldsearch.php). Two GWASs in the CATIE sample have been published.[17,18] However, an association of the *DPP6* gene SNP with TD has not been reported. This may be due to differences in GWAS design, TD definition and/or ethnicity between studies.

In addition to the GWASs in the CATIE sample,[17,18] an association of the SNP rs3943552 in the GLI2 gene with TD

was independently supported in Jewish Israeli schizophrenia patients of Ashkenazi origin.[18] A large candidate gene study of TD based on CATIE was also reported.[27] We were able to evaluate associations in the current Japanese screening sample between TD and 24 SNPs that were among the top results observed in the CATIE sample. Five SNPs were associated with TD with nominal significance and all alleles were in the same direction of risk between the CATIE and Japanese samples (Supplementary Table 1). These findings indicate common SNPs associated with TD beyond ethnicity as well as promising SNPs for further investigation.

In our previous studies, we searched for associations between SNPs on the Illumina Human-1 Genotyping 109K BeadChip and TD.[19] We selected 63 SNPs with allelic *P*-values <0.002 and located within 10 kb from known genes for subsequent replication analysis. One SNP, rs1047053, which is located in the 3′-untranslated region of the *DPP6* gene, was included among the top 63 SNPs; however, the association was not replicated. The second most significant association for the SNPs in the *DPP6* gene on the Illumina Human-1 Chip was for rs2052218, which is separated from rs6977820 by approximately 14 kb. However, allelic $P=0.003$ was just outside the criteria for the replication analysis in the previous study. Thus, we did not further examine the association. In this study, we searched for associations by using the HumanHap370 BeadChip. Most of the subjects (100 out of 122) were the same as those studied using the Human-1 BeadChip. However, a small number of SNPs (14 662 SNPs) overlapped and the SNPs of rs2445142, rs4738269 and rs2061051 SNPs were not included in the HumanHap370 BeadChip. The rs1080333 and rs2919415 SNPs on the HumanHap370 BeadChip, which is in LD with rs4738269 in the *KCNB2* gene, were able to be analyzed again and showed almost the same allelic *P*-value with TD ($P=0.0005$).

The *DPP6* gene is preferentially expressed in neurons that contain predominantly Kv4 (hippocampal pyramidal neurons, striatal medium spiny neurons and cerebellar granule cells).[28] DPP6 is well known as an auxiliary subunit of the Kv4 channels in CNS neurons, although it may have additional Kv4-unrelated functions in the brain.[29] Without DPP6, the Kv4 channels inactivate more slowly and recover more slowly from inactivation than the channels in neurons.[30,31] DPP6 is required to efficiently traffic the Kv4 channels to the plasma membrane and regulate the functional properties of the channels, and may also be important in determining the localization of the channels to specific neuronal compartments, their dynamics and their response to neuromodulators.[32] The transient potassium current mediated by Kv4 channels is a common target of dopamine modulation in most cell types.[33] Chronic haloperidol treatment upregulates dopamine neuron Kv4.3mRNA and an increased number of functional A-type K+ channels causes a decreased intrinsic firing of dopamine neurons elicited by chronic haloperidol.[34] In this study, we observed that expression of *Dpp6* was increased by long-term administration of haloperidol. Increased DPP6 may lower the pacemaker frequency of dopamine release, which decreases sensitivity to dopamine. Therefore, we hypothesized that lower levels of *DPP6* found in people with the rs6977820 risk genotype may be prone to dopamine super-sensitivity when long-term blockade of the dopamine D2 receptor produces hypersensitivity to dopamine in DRD2.

Several limitations in this study should be mentioned. The biggest weakness is the small sample size. It is difficult to find a large number of subjects who have suffered from treatment-resistant TD. Further replication is necessary. Furthermore, although the identified SNP was associated with the mRNA levels of *DPP6*, the mechanism for the association has not been clarified. We only analyzed human prefrontal cortex brain and did not analyze mice showing viscous chewing induced by haloperidol only.

The present study implicates *DPP6* in susceptibility to TD. However, it does not appear to be the sole genetic determinant. GWAS studies including ours suggest that the genetic nature of susceptibility to TD is multi-factorial inheritance.

Conflict of interest

The authors declare that no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Patient Report

Case of glycogen storage disease type VI (phosphorylase deficiency) complicated by focal nodular hyperplasia

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Key words focal nodular hyperplasia, glycogen phosphorylase, glycogen storage disease.

Although it is well known that hepatic tumors often develop in patients with glycogen storage disease (GSD) types Ia and III, the formation of these tumors has not been reported in other forms of hepatic GSD. In this report, a patient with GSD type VI (phosphorylase deficiency; OMIM 232700) complicated with a hepatic benign tumor, focal nodular hyperplasia (FNH), is presented. This case indicates that regular check-ups for hepatic tumors are necessary, not only in patients with GSD types Ia or III, but also in patients with other forms of hepatic GSD.

Case Report

A female patient was referred to our hospital when she was 5 years of age for further investigation of hepatomegaly, which had been detected when she had visited a clinic when she was 5 years old. She was born to healthy non-consanguineous parents and had no history of hypoglycemia or nasal bleeding. On physical examination, her height was 101 cm (−1.5 SD) and her bodyweight was 16 kg (−1.0 SD). The liver was firm and palpable 7 cm below the right costal margin, whereas the spleen was not palpable. The results of a fasting blood test collected at that time were as follows: aspartate aminotransferase 37U/L, alanine aminotransferase 24U/L, blood glucose 85 mg/dL, lactate 6.2 mg/dL, uric acid 5.9 mg/dL, total cholesterol 229 mg/dL and triglyceride 88 mg/dL. A plain abdominal computed tomography (CT) scan showed an enlarged liver with a density considerably higher than that of the spleen (CT values: liver, 80; spleen, 42) (Fig. 1). Glucose and galactose loading tests were performed. The serum lactate level was not elevated when glucose was loaded, although it increased to a maximum of 56 mg/dL one hour after loading (normal <35 mg/dL). A glucagon loading test was performed after a 15-h fast, with the serum glucose level increasing from 71 to 128 mg/dL one hour after loading. On the basis of these data, GSD was suspected and accordingly the enzyme activities of hepatic GSD, that is, debranching enzyme, phosphorylase and phosphorylase b kinase, were measured in peripheral blood. The results of all these tests were normal (Table 1). Informed consent for a liver needle biopsy for measurement of enzyme activity was not obtained. Although the enzyme activity of phosphorylase b kinase measured in peripheral blood was normal, a tentative diagnosis of GSD type IX (phosphorylase b kinase deficiency) was made based on the physical, laboratory and radiological findings and the results of the loading tests. Regular check-ups including abdominal CT scans for potential formation of hepatic tumor were performed every year. The patient's growth curve showed that she attained mean values around the time of puberty. The results of blood tests obtained between 5 and 14 years of age were as follows (mean ± SD): uric acid 5.9 ± 0.6 mg/dL, total cholesterol 208 ± 21.0 mg/dL and triglyceride 198 ± 111 mg/dL.

When the patient was 15 years of age, the early phase of a contrast-enhanced abdominal CT scan revealed an enhanced lesion in the liver (Fig. 1). After obtaining informed consent, specimens were obtained by needle biopsy from the tumor and non-tumor part of the liver. Histological findings of the non-tumor specimen showed strong periodic acid-Schiff (PAS) staining in hepatocytes that disappeared following diastase treatment, findings compatible with GSD. Histology of the tumor specimen demonstrated pericellular fibrosis, compatible with the diagnosis of FNH (Fig. 2). Fibrous bands containing bile ductules were not observed in the specimens. Enzyme activities of hepatic GSD were measured using liver tissue from the non-tumor section, which revealed that phosphorylase enzyme activity was 2.3 nmol/min/mg protein, a value corresponding to 24% of normal. The enzyme activity of both debranching enzyme and phosphorylase b kinase was normal (Table 1). Informed consent for gene analysis of phosphorylase (*PGYL*) could not be obtained. We concluded that the patient's diagnosis was GSD VI (phosphorylase deficiency) complicated by FNH. We elected to forego surgical treatment in favor of long-term observation. The size of the tumor has been monitored regularly with ultrasonography. As of now, the tumor does not appear to be enlarging.

Discussion

In this report we present a patient with GSD type VI complicated by FNH. This is the first report of a hepatic tumor complication

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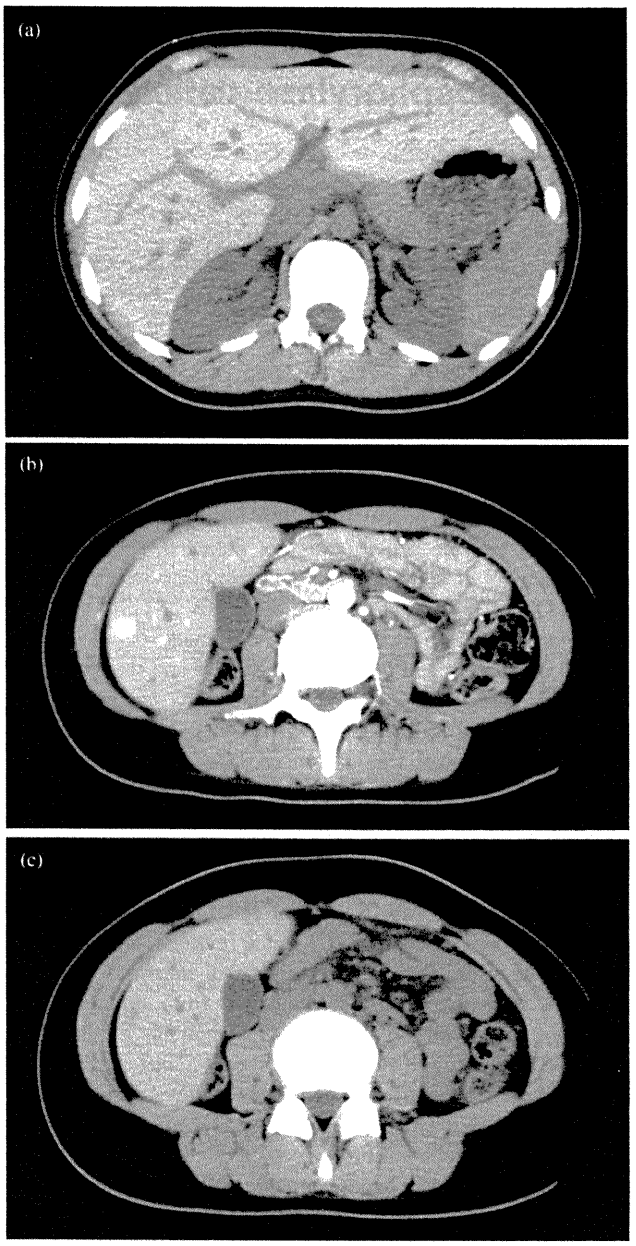

Fig. 1 (a) The findings of a plain abdominal computed tomography (CT) scan at 5 years of age. The CT value of the liver was markedly elevated compared with that of the spleen and kidneys. (b) The findings of the early phase of a contrast-enhanced abdominal CT scan at 15 years of age showing an enhanced lesion. (c) The findings of the same section as (b), without contrast enhancement.

in a patient with GSD type VI, a different hepatic form of GSD than types Ia or III. As hepatic tumors are often found in patients with GSD types Ia and III, regular check-ups for these tumors are performed routinely in these patients. However, this report indicates that regular check-ups for hepatic tumor are also necessary in patients with hepatic forms of GSD other than types Ia or III.

In patients with GSD type Ia, hepatic adenoma is the most common tumor described; however other tumors, including hepatocellular carcinoma (HCC),[1] described in patients with GSD III,[2] hepatoblastomas,[3] and FNH[4] have also been reported.

Hepatic adenomas are a benign tumor, consisting of a nodular proliferation of hepatocytes arranged in cords having no relationship to portal tracts. They often have a pushing border abutting against the surrounding liver. The hepatic adenoma has, on rare occasions, been known to progress to HCC,[1] and this is one of the most important reasons why regular check-ups and follow up after the discovery of an adenoma are necessary in a patient with GSD Ia. FNH is typically a single mass in an otherwise healthy liver characterized by central scarring that radiates between multiple nodules of regenerating parenchyma. Like the hepatic adenoma, it is also a benign tumor parenchyma but the potential for malignant transformation of FNH into HCC has not been demonstrated. However, a case of HCC arising within FNH has been reported recently[5] and this report emphasizes the importance of detecting FNH, even though the FNH itself is benign.

The mechanism of tumor formation in GSD type Ia is considered to occur by the following sequence.[6] Increased amounts of free fatty acids are released from adipose tissue, taken up by the liver and channeled into triglyceride formation. Malonyl-CoA is a key lipogenic intermediate in this process, which, in turn, causes inhibition of carnitine palmitoyltransferase I and limitation of mitochondrial beta-oxidation. This results in fatty acids being more likely to be channeled into extramitochondrial pathways, such as within peroxisomes, leading to an increase in hydrogen peroxide generation. This results in increased generation of free radicals that are capable of inflicting direct DNA damage, which may initiate the development of hepatic tumors. Although the patient reported here was diagnosed with GSD type VI, hypertriglyceridemia was almost always observed during the clinical course of the disease, similar to that seen in cases with type I GSD. We anticipate this would have resulted in increased generation of free radicals by the mechanism described above and could possibly have caused the formation of FNH we observed in the patient.

In our patient we observed a difference in phosphorylase activity between peripheral blood and liver tissue. Three isoforms of phosphorylase exist, that is, liver, brain and muscle. As the liver isoform is expressed in peripheral blood,[7,8] phosphorylase activity in peripheral blood and the liver should be the same. The reason why phosphorylase activity in peripheral blood and liver was different in our patient is not clear, although similar findings have been reported elsewhere.[9] Mutation analysis of the liver glycogen phosphorylase gene (*PYGL*) is necessary for further confirmation of this diagnosis.

In summary, we report a patient with GSD VI complicated with FNH. This case indicates that regular check-ups for hepatic tumors are necessary, not only in patients with GSD types Ia or III, but also in patients with other forms of hepatic GSD.

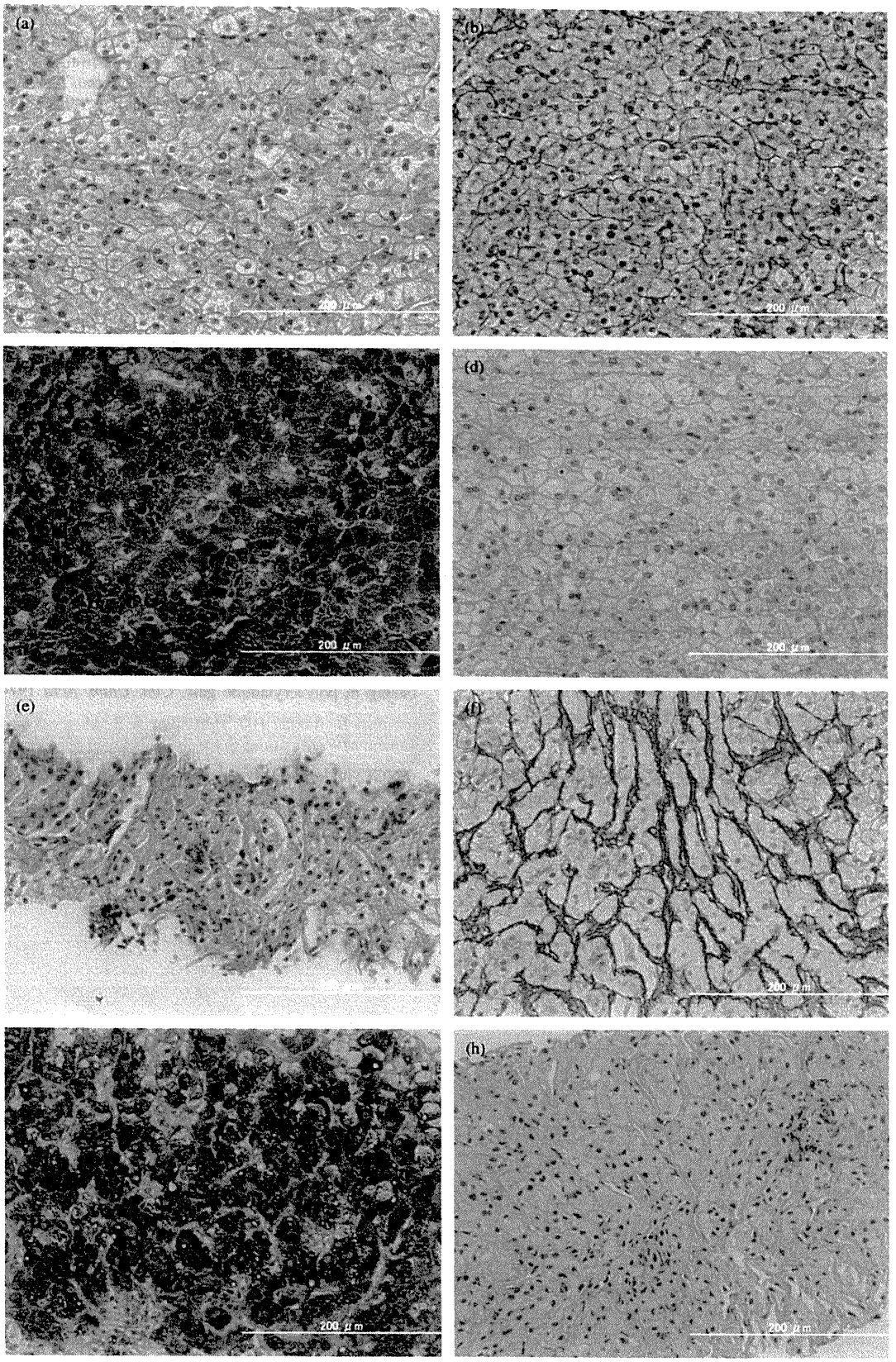

Fig. 2 Histological findings of the liver from (a–d) non-tumor and (e–h) tumor specimens. (a and e) Hematoxylin–eosin (HE) stain, (b and f) silver staining, (c and g) periodic acid-Schiff (PAS) staining and (d and h) PAS staining after diastase treatment. In the non-tumor specimen, the hepatocytes had (a) clear cytoplasm with (b) no fibrosis observed. (c and d) All the hepatocytes were stained strongly by PAS, which disappeared following diastase treatment. (e and f) In tumor specimens, pericellular fibrosis was observed, whereas fibrous bands in which bile ductules were proliferating were not. On the basis of the finding of pericellular fibrosis, a diagnosis of focal nodular hyperplasia was made. The original magnification was ×20.

Table 1 Results of enzyme activity measurements in the patient and controls

Peripheral blood	Patient	Control 1	Control 2	
Debranching enzyme	14.8	24.9	19.1	Nmole glucose/hour/mg
Phosphorylase	6.3	6.1	7.2	Nmole/min/mg
Phosphorylase b kinase	45.8	44.5	42.0	Nmole/min/g Hb
Liver	**Patient**	**Controls**		
Debranching enzyme	243.4	197.4 ± 32.8 (n = 10)		Nmole glucose/hour/mg
Phosphorylase	2.3	9.6 ± 1.7 (n = 10)		Nmole/min/mg
Phosphorylase b kinase	49.6	62.7 ± 11.8 (n = 9)		Nmole/min/mg

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Original article

Study of *HOXD* genes in autism particularly regarding the ratio of second to fourth digit length

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Abstract

Multiple genes are involved in the pathogenesis of autism. To study the causative gene, the relationship between autism endophenotypes and their closely related genes has been analyzed. There is a subgroup of autism spectrum disorder (ASD) in which the ratio of second digit length to fourth digit length (2D/4D) is low (short digit group, SDG). We studied the relationship between ASD and *HOXD* genes, which are located in the candidate locus for ASD and are associated with digit morphogenesis, with a particular focus on SDG. We analyzed 25 SNPs of *HOXD11*, *HOXD12*, and *HOXD13* in the subject of 98 ASD, 89 healthy controls, and 16 non-autistic patients (non-ASD). There was no significant difference in the genotype frequencies between the ASD and the healthy controls. However, the G-112T heterozygote in the promoter region of *HOXD11* was observed in only four patients with ASD and in none of the healthy controls or non-ASD subjects. Moreover, this *HOXD11* G-112T was observed in three of 11 SDG with ASD but in none of the 15 non-SDG patients with ASD. There were eight SDG patients among the non-ASD ones, but this polymorphism was observed in none of them. Considering the above results, it is expected that candidate genes will be further identified, using *HOXD11* G-112T polymorphism as a marker, by analyzing genes located near 2q in a larger number of ASD subjects with clinical signs of SDG.

Keywords: Autism; *HOXD*; 2D/4D; Endophenotype; Genetic polymorphism

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1. Introduction

Autism is basically characterized by severely impaired social interaction and communication, and a limited range of activities and interests. As the diagnosis of autism is made on the basis of patients' behavioral characteristics, the disorder is not caused by only one factor. It is considered that various genetic and environmental factors are involved in the occurrence of autism, and their interactions are complex. In 1998, the International Molecular Genetic Study of Autism Consortium (IMGSC) reported their genome-wide linkage analysis of families in which there was more than one member with idiopathic autism [1]. On the basis of the results of a subsequent large-scale genome-wide scan, candidate

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gene loci, including 7q21.2–q36.2, 16p12.1–p13.3, 6q14.3–q23.2, 2q24.1–q33.1, 17q11.1–q21.2, 1q21–q44, and 3q21.3–q29, were identified [2]. In an attempt to increase the linkage, a nearly homogeneous group was selected among patients with autism of heterogeneous causes. Autism patients were classified into subgroups or subsets in accordance with the phenotype of autism [3], such as through a quantitative trait locus (QTL) analysis of the constituent elements of endophenotypes in autism [4], and an ordered-subset analysis [5] was carried out. The ratio of second digit (2D) length to fourth digit (4D) length (2D/4D) is very low in some autism patients [6,7]. The *homeo box D (HOXD)* gene family is involved in skeletal morphogenesis, and correlations between digit length and the expression levels of *HOXD11*, *HOXD12*, and *HOXD13* have been observed [8,9]. In addition, *HOXD* genes form a cluster at 2q24.1–q33.1, which has been found to be a candidate locus by a genome-wide scan [3]. Therefore, we considered that digit length is one of the small physical signs of autism. Hence, we investigated the relationships between autism and polymorphism of *HOXD11*, *HOXD12*, and *HOXD13*. Moreover, we classified autism patients into two categories: patients with a low 2D/4D formed the short digit group (SDG), while the remaining patients formed the non-short-digit group (non-SDG). We also examined the genetic polymorphism of these three genes between SDG and non-SDG with autism and also between SDG with and without autism. No analysis of autism focusing on these relationships has been reported to date.

2. Subjects and methods

Seven patients with autism in the SDG were screened for the presence or absence of gene mutations in the exon and intron of *HOXD11*, *HOXD12*, and *HOXD13*, and for gene polymorphisms. The genotypic frequencies of the detected polymorphisms and the polymorphisms already listed in the GenBank were compared between the autism patients and the controls. Finally, the genotypes of the above polymorphisms of the autism patients in SDG were investigated.

2.1. Subjects

The subjects examined by genetic analysis in this study were 98 patients who visited the Department of Pediatrics, Hamamatsu University School of Medicine and Hamamatsu City Medical Center for Developmental Medicine, and who were diagnosed as having autism, PDD-NOS, and Asperger syndrome on the basis of the criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV [10]). Patients with clear underlying diseases such as chromosomal abnormalities, tuberous sclerosis, and Fragile X syndrome were excluded from the study. The patients were of 82 males and 16 females with ages ranging from 5 years and 2 months to 31 years and 10 months (mean age: 12 years and 7 months). In terms of ethnicity, 95 patients had Japanese parents, 2 had Japanese fathers and Filipino mothers, and 1 had Bangladeshi parents. Eighty-nine subjects without any neurological abnormality served as healthy controls for gene analysis; all of them were Japanese and their sex and age were not determined. Thirty patients were also examined as disease controls, including 16 non-autistic patients, 14 mentally retarded patients, and 2 AD/HD patients, all of whom were Japanese.

2.2. Measurement of second and fourth digit lengths

A digital camera providing three-megapixel images was used for the measurement of the 2D and 4D lengths. Each subject's right hand was placed palm-up on a flat desk, and was photographed with the camera 20 cm above the hand. Three pediatric neurologists separately measured the 2D and 4D lengths from the line of the base to the tip of the digits three times using the image analyzing software Scion Image (NIH). The mean ratio of 2D length to 4D length (2D/4D) was calculated. In this study, patients with lower than the mean 2D/4D of the autism patients reported by Osawa et al., that is, a 2D/4D of 0.94 or lower, were classified as SDG [7].

2.3. Gene analysis

Seven patients with autism (6 males and 1 female) in the SDG were screened for the presence or absence of gene mutations and gene polymorphisms by the direct sequencing method. *HOXD11*, *HOXD12*, and *HOXD13* – each consisting of two exons and one intron – were searched for in a region from approximately 500 bp upstream, including a promoter, to approximately 500 bp downstream of the gene. Genomic DNA extracted from lymphocytes using a DNA extraction kit (Takara Co., Shiga, Japan) was used. DNA was amplified by PCR using a Taq PCR Core kit (QIAGEN Co., CA, USA), and the base sequence was obtained by the direct sequencing method. Genotypes were determined for single nucleotide polymorphism (SNP) in five loci that were newly found by this method in this study and for SNP in 20 loci that are listed in the online database GenBank (NCBL dbSNP). Genotypes in some loci were also determined by real-time PCR analysis using a TaqMan allelic discrimination assay (Applied Biosystems).

2.4. Statistical analysis

Genotypic frequency and allelic frequency of the autism patients were compared to those of the healthy con-

trol group using a χ^2 test or Fisher's exact test with SPSS 12.0J for a Windows-based System. A statistical significance level of $p \leqq 0.05$ was set.

3. Results

2D/4D was determined in 28 patients (24 males and 4 females) out of the 98 autism patients. Eleven patients (9 males and 2 females) of these 28 patients were classified as SDG. The clinical features of these patients, including sex, age, and the severity of mental retardation, are shown in Table 1. A high percentage of patients with severe mental retardation were observed in SDG with autism, whereas no patients with severe mental retardation were observed in non-SDG with autism. We also measured 2D/4D in 16 non-autistic patients in the disease control group, and 8 patients were classified as SDG and 8 patients as non-SDG. The results of the 2D/4D values of the 28 ASD and 30 non-ASD patients are shown in Fig. 1.

The results of the polymorphism analysis are shown in Table 2. No significant difference in polymorphism was observed between the autism patients and the healthy control group. However, with regarding to SNP in the promoter region of *HOXD11* G-112T, heterozygosity was observed in 4 autism patients, but not in the healthy or disease control group. The SNP in the promoter region of *HOXD12* -C226A and the SNP in

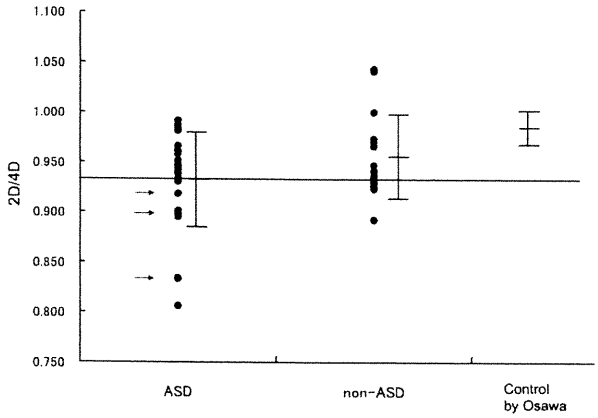

Fig. 1. 2D/4D values for the ASD28 cases and the non-ASD30 cases. Mean ± SD was presented. The M-ASD line is the average for the ASD cases; at or below this line is the SDG. As a reference, we showed the mean ± SD for normally healthy children as calculated by Osawa et al. [7]. The arrow indicates cases with *HOXD11* heterogeneity.

Table 1
Clinical features of patients.

	All the autistic disorder patients	Patients with 2D/4D determined		
		Total	SDG	NSDG
Number of patients	98	28	11	17
Sex				
Males:females	82:16 (5.1:1)	24:4 (5.5:1)	9:2 (4.5:1)	15:2 (7.5:1)
Age	5 y 2 m–31 y 10 m	5 y 4 m–31 y 10 m	8 y 1 m–31 y 10 m	5 y 4 m–16 y 7 m
Median	11 y 6 m	12 y 0 m	14 y 4 m	9 y 2 m
Mean	12 y 7 m	12 y 11 m	16 y 6 m	10 y 4 m
Family history: (3 generations)				
With[a]	22 (22.4%)	10 (35.7%)	3 (30.0%)	7 (41.2%)
Those with autism	7 (7.3%)	5 (17.9%)	2 (20.0%)	3 (17.4%)
Without	69 (70.4%)	16 (57.1%)	7 (70.0%)	7 (41.2%)
Mental retardation				
Without	10 (10.3%)	7 (25.0%)	2 (18.2%)	5 (29.4%)
Minor	21 (21.6%)	6 (21.4%)	2 (18.2%)	4 (23.5%)
Moderate	44 (45.4%)	10 (35.7%)	2 (18.2%)	8 (47.1%)
Severe	22 (22.7%)	5 (17.9%)	5 (45.5%)	0
Age at walk alone	9–48 m (91 cases)	9–48 m (26)	11–48 m (10)	9–18 m (16)
Median	13 m	12 m	12 m	12 m
Mean	13.9 m	14.3 m	18 m	12.9 m
Age at first word	10 m–6 y 10 m (80 cases)	11 m–6 y 10 m (25)	11 m–6 y 10 m (10)	1 y 3 m–3 y 5 m (15)
Median	1 y 6 m	1 y 6 m	1 y 6 m, 1 y 11 m	1 y 10 m
Mean	1 y 9 m	2 y 1 m	2 y 4 m	1 y 11 m
No. of patients 2 y or over	28	10	4	6
Age at first phrase	1 y 6 m–5 y 0 m (31 cases)	1 y 7 m–5 y 0 m (13)	2 y 6 m–5 y 0 m (5)	1 y 7 m–4 y 0 m (8)
Median	2 y 11 m	2 y 11 m	3 y 0 m	2 y
Mean	2 y 10 m	2 y 9 m	3 y 2 m	2 y 5 m
No. of patients 3 y or over	16	5	3	2

[a] Family history with psychiatric disorders including major depression, autism etc.

Table 2
Results of analysis of gene polymorphisms.

Gene	Location in gene	dtSNP ID	Allele	Frequency		Genotype	Frequency	
				Autism	Control		Autism	Control
HOXD11	Promoter		G	0.979	1	GG	0.959	1
			T	0.021	0	GT	0.042	0
						TT	0	0
	Intron	rs84746	A	0.711	0.721	AA	0.571	0.561
			C	0.289	0.288	AC	0.230	0.371
						CC	0.133	0.067
	Exon 2	rs863678	G	0.541	0.567	GG	0.316	0.292
			T	0.459	0.443	GT	0.449	0.551
						TT	0.235	0.157
	Exon2	rs6745764	A	0.214	0.18	AA	0.031	0.011
			G	0.786	0.82	AG	0.367	0.337
						GG	0.602	0.652
HOXD12	Promoter		A	0.041	0.028	AA	0	0
			C	0.959	0.972	AC	0.082	0.056
						CC	0.918	0.944
	Promoter		G	0.929	0.955	GG	0.929	0.955
			T	0.071	0.045	GT	0.071	0.045
						TT	0	0
	Exon 1	rs847151	A	0.041	0.028	AA	0	0
			G	0.959	0.972	AG	0.082	0.056
						GG	0.918	0.944
HOXD13	Promoter	rs847196	C	0.893	0.938	CC	0.786	0.876
			G	0.107	0.061	CG	0.214	0.124
						GG	0	0
	Promoter		A	0.082	0.107	AA	0	0
			T	0.918	0.893	AT	0.163	0.213
						TT	0.837	0.787
	Exon 1		C	0.985	0.989	CC	0.969	0.978
			T	0.015	0.011	CT	0.031	0.022
						TT	0	0
	Exon 1	rs2518053	A	0.408	0.455	AA	0.173	0.235
			G	0.592	0.545	AG	0.469	0.438
						GG	0.357	0.326
	Intron	rs847194	A	0.684	0.657	AA	0.459	0.404
			C	0.316	0.343	AC	0.449	0.506
						CC	0.092	0.09

SNP with no polymorphism detected in the present cases analyzed among the SNPs listed at the GenBank

HOXD11	Promoter	rs2736846	*HOXD13*	Exon 1	rs847195
	Intron	rs2736847		Exon 1	rs13392701
	Exon 2	rs12995279		Intron	rs847193
	Exon2	rs12995280		Intron	rs847192
HOXD12	Exon 1	rs2551807		Exon 2	rs28928892
	Exon2	rs2553776		Exon 2	rs28933082
				Exon 2	rs28928891

exon 1 of *HOXD12* (rs847151, G364A) showed a nearly complete linkage disequilibrium. Heterozygosity for both *HOXD12* -C226A and *HOXD12* G364A was observed in five healthy controls and eight autism patients. Furthermore, all of the five controls heterozygous for *HOXD12* -C226A and *HOXD12* G364A were homozygous for *HOXD11* -G112G. On the other hand, of the eight autism patients heterozygous for both *HOXD12* -C226A and *HOXD12* G364A, four were homozygous and four were heterozygous for *HOXD11* G-112T. Taken together, heterozygosity in all the three loci *HOXD11* G-112T, *HOXD12* -C226A, and *HOXD12* G364A was found in four autism patients but not in the healthy controls. Table 3 shows the relationships between the polymorphisms in these three loci for two cases: SDG and non-SDG with autism and SDG and non-SDG without autism. Of the four patients heterozygous for *HOXD11* G-112T, three in whom digit length was measured were classified into SDG with autism and the rest was unknown. The clinical type of ASD of the patients with having *HOXD11* heterogeneity was classified as autistic disorder in all cases. No patients