Table 2 Genotypic and Allelic Distributions for SNPs in the *RELA* Gene Between Patients in Schizophrenia and Controls

(a) In males

Marker		M/m	Location	SCZ (N = 623)			CON (N = 773)			Genotypic p value (df = 2)	SCZ	CON	Allelic p value (df = 1)	OR (95%CI)
SNP ID	Position[a]			M/M	M/m	m/m	M/M	M/m	m/m		MAF			
SNP1	10728386	C/T	Intron 10	0.35	0.47	0.18	0.28	0.48	0.23	**0.014**	0.42	0.48	**0.0030**	0.79 (0.68–0.92)
SNP2	10730962	G/C	Intron 8	0.76	0.23	0.01	0.75	0.24	0.01	0.88	0.12	0.13	0.69	0.95 (0.76–1.20)
SNP3	10733141	G/A	Intron 5	0.34	0.47	0.19	0.28	0.48	0.24	**0.0080**	0.42	0.48	**0.0016**	0.78 (0.67–0.91)
SNP4	10735731	C/T	Intron 1	0.30	0.48	0.22	0.38	0.45	0.16	**0.0022**	0.46	0.39	**0.00037**	1.32 (1.13–1.54)

(b) In females

Marker		M/m	Location	SCZ (N = 601)			CON (N = 890)			Genotypic p value (df = 2)	SCZ	CON	Allelic p value (df = 1)	OR (95%CI)
SNP ID	Position[a]			M/M	M/m	m/m	M/M	M/m	m/m		MAF			
SNP1	10728386	C/T	Intron 10	0.30	0.50	0.20	0.30	0.48	0.23	0.52	0.45	0.47	0.45	0.94 (0.81–1.10)
SNP2	10730962	G/C	Intron 8	0.80	0.19	0.01	0.77	0.22	0.01	0.35	0.11	0.12	0.16	0.84 (0.67–1.07)
SNP3	10733141	G/A	Intron 5	0.30	0.49	0.21	0.29	0.47	0.24	0.52	0.45	0.47	0.32	0.93 (0.80–1.08)
SNP4	10735731	C/T	Intron 1	0.31	0.49	0.20	0.34	0.49	0.16	0.13	0.45	0.41	0.054	1.16 (1.00–1.35)

CI, confidence interval; CON, controls; M, major allele; m, minor allele; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism; SCZ, patients with schizophrenia.
All the alleles are represented according to the minus strand DNA sequence.
P values <0.05 are in bold and underlined.
[a]db SNP build 129.

only in the Japanese population (SNP1: $r^2 = 0.27$, $p = 0.0003$; SNP3: $r^2 = 0.25$, $p = 0.0005$; SNP4: $r^2 = 0.15$, $p = 0.0089$). Interestingly, the risk alleles in all SNPs were associated with lower gene expression (SNP1: C allele, SNP3: G allele, SNP4: T allele).

Because SNP4 is located in intron 1, this SNP is possibly related to the regulation of gene transcription. An *in silico* search for potential transcription factor binding sites in the sequences surrounding SNP4 by the Patch 1.0 program showed that SNP4 could potentially alter androgen receptor (AR) binding. The consensus sequence of the AR binding site is AAAA<u>C</u>T (C allele of SNP4 is a non-risk allele). Thus, the mismatch in the AR consensus sequence created by the single nucleotide change of SNP4 (C allele to risk T allele: AAAA<u>T</u>T) could lead to lower transcriptional activity of the *RELA* gene, which is consistent with *in silico* expression data and genetic association results in male.

The Effect of *RELA* Genotypes on PPI

We examined possible associations between the genotype of four SNPs and PPI in patients with schizophrenia, as PPI is one of physiological phenotypes of schizophrenia. There was no difference in the demographic variables, including age, sex, current smoking status, years of education, chlorpromazine equivalents of total anti-psychotics, age at onset, duration of illness, medicated years with anti-psychotics, acoustic startle reflex, and habituation, between

genotype groups, except for the chlorpromazine equivalents of total anti-psychotics of SNP1 ($p = 0.028$) and SNP3 ($p = 0.043$) (Supplementary Table S2). One-way ANCOVA showed significant effects of the four genotypes on at least one of the PPI conditions (Table 5). The SNPSpD correction for multiple SNPs tested, revealed the association between three at risk genotypes (SNP1, SNP3, and SNP4) and PPI (SNP1 PPI86: corrected $p = 0.033$, PPI90: corrected $p = 0.044$; SNP3 PPI86: corrected $p = 0.036$, PPI90: corrected $p = 0.044$; SNP4 PPI82: corrected $p = 0.019$) (Table 5, Figure 2). However, the effects of the non-risk SNP (SNP2) on PPI was no longer significant after the SNPSpD correction (PPI86: corrected $p = 0.083$) (Table 5, Figure 2). The patients with the C/C at risk genotype of SNP1, patients with the G/G at risk genotype of SNP3 and patients with the T/T at risk genotype of SNP4 showed significant deficits in PPI.

DISCUSSION

In this study, we first provided evidence that genetic variants of the *RELA* gene are associated with the risk for schizophrenia. Next, *in silico* analysis suggested that the risk SNPs in the *RELA* gene might be associated with gene expression differences in lymphoblasts. Finally, we measured the effects of the *RELA* genotypes on PPIs in patients with schizophrenia. Our results indicated that the risk alleles were associated with reduced PPIs.

Table 3 Haplotype Analysis of the *RELA* Gene Between Patients and Controls

Haplotype[a]	Frequency		Individual p (χ^2)	Global p (χ^2)
	Patients	Controls		
SNP1-SNP2				**0.0019 (12.13)**
1–0	0.43	0.47	**0.012 (6.88)**	
0–0	0.45	0.40	**0.00060 (12.10)**	
0–1	0.12	0.13	0.19 (1.59)	
SNP2-SNP3				**0.00080 (13.24)**
0–1	0.44	0.48	**0.0053 (8.03)**	
0–0	0.45	0.40	**0.00030 (13.24)**	
1–0	0.12	0.13	0.23 (1.38)	
SNP3-SNP4				**0.00040 (15.14)**
1–0	0.43	0.48	**0.0031 (9.19)**	
0–1	0.45	0.40	**0.00006 (15.14)**	
0–0	0.12	0.13	0.21 (1.56)	
SNP1-SNP2-SNP3				**0.0022 (12.03)**
1–0–1	0.43	0.47	**0.0071 (7.07)**	
0–0–0	0.45	0.40	**0.00070 (12.02)**	
0–1–0	0.12	0.13	0.22 (1.40)	
SNP2-SNP3-SNP4				**0.00090 (13.66)**
0–1–0	0.44	0.48	**0.0028 (8.68)**	
0–0–1	0.45	0.40	**0.00040 (13.65)**	
1–0–0	0.11	0.12	0.26 (1.20)	
SNP1-SNP2-SNP3-SNP4				**0.0022 (13.11)**
1–0–1–0	0.43	0.47	**0.0058 (8.32)**	
0–0–0–1	0.45	0.40	**0.00060 (13.11)**	
0–1–0–0	0.12	0.12	0.27 (1.17)	

0, major allele; 1, minor allele. Haplotypes with frequencies of <3% in each group were excluded.
Significant p values <0.05 are represented by bold faces and underlines.

Table 4 Association Between the *RELA* Gene SNPs and mRNA Expression in a Japanese Population

SNP	Location	Population	r	Beta	SE	t	p
SNP1	Intron 10	JPT	0.52	0.24	0.06	3.91	**0.0003**
		CHB	−0.05	−0.02	0.06	−0.30	0.76
		CEU	−0.07	−0.03	0.05	−0.52	0.61
		YRI	0.15	0.05	0.05	1.16	0.25
SNP3	Intron 5	JPT	−0.50	−0.23	0.06	−3.74	**0.00050**
		CHB	0.05	0.02	0.06	0.30	0.76
		CEU	0.07	0.03	0.05	0.51	0.61
		YRI	−0.13	−0.05	0.05	−1.01	0.32
SNP4	Intron 1	JPT	−0.39	−0.17	0.06	−2.74	**0.0089**
		CHB	0.11	0.04	0.06	0.74	0.47
		CEU	−0.01	0.00	0.05	−0.06	0.95
		YRI	−0.16	−0.05	0.04	−1.20	0.24

JPT, Japanese in Tokyo, Japan; CHB, Han Chinese in Beijing, China; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection (Parent); YRI, Yoruban in Ibadan, Nigeria (Parent).
P values <0.05 are in bold and underlined.

Table 5 Association Between PPI and *RELA* Genotypes in Patients with Schizophrenia

SNP ID	PPI		P value (F value)	Corrected P	Cohen's d
SNP1	C/C	T carrier			
PPI82	17.0 ± 8.8	37.1 ± 4.4	**0.024 (5.39)**	0.066	−0.63
PPI86	14.1 ± 12.1	38.9 ± 4.6	**0.012 (6.75)**	**0.033**	−0.61
PPI90	24.2 ± 8.0	42.4 ± 4.8	**0.016 (6.25)**	**0.044**	−0.58
SNP2	G/G	C carrier			
PPI82	31.5 ± 5.1	28.1 ± 6.6	0.95 (<0.01)	>0.99	0.12
PPI86	38.0 ± 4.5	7.0 ± 15.1	**0.030 (4.97)**	0.083	0.73
PPI90	41.9 ± 4.8	18.2 ± 7.7	0.072 (3.38)	0.20	0.82
SNP3	G/G	A carrier			
PPI82	17.0 ± 8.8	36.5 ± 4.4	**0.029 (5.06)**	0.080	−0.62
PPI86	14.1 ± 12.1	39.3 ± 4.3	**0.013 (6.73)**	**0.036**	−0.64
PPI90	24.2 ± 8.0	42.7 ± 4.7	**0.016 (6.30)**	**0.044**	−0.61
SNP4	T/T	C carrier			
PPI82	8.6 ± 12.5	36.6 ± 4.0	**0.0068 (8.00)**	**0.019**	−0.81
PPI86	22.3 ± 10.2	32.9 ± 6.0	0.35 (0.89)	0.97	−0.29
PPI90	21.4 ± 11.5	40.2 ± 4.5	**0.041 (4.40)**	0.11	−0.56

SNP1: C/C, patients with C/C genotype ($n = 17$); T carrier, patients with C/T or T/T genotype ($n = 37$). SNP2: C carrier, patients with C/C or C/G genotype ($n = 12$); G/G, patients with G/G genotype ($n = 42$). SNP3: G/G, patients with G/G genotype ($n = 17$); A carrier, patients with G/A or A/A genotype ($n = 35$). SNP4: T/T, patients with the T/T genotype ($n = 11$); C carrier, patients with the T/C or C/C genotypes ($n = 42$). Means ± SE are shown. The effects of the *RELA* genotypes on PPI were analyzed by ANCOVA with age, sex, and current smoking status as covariates.
P values <0.05 are in bold face and underline. SNPSpD correction was applied to correct for multiple SNPs tested (the effective number of independent marker loci: 2.76).

As the association of the *RELA* gene and schizophrenia was supported by a number of statistical analyses such as genotypic and allelic associations for four SNPs (total 28) and haplotype analysis (total 24), the correction for multiple testing should be considered. In this study, overall genetic association tests were 52; however, all tests were not independent and multiple hypotheses were included. Thus, Bonferroni correction, a method to correct for multiple independent tests for one hypothesis, might not be appropriate. The consensus how to correct such multiple testing has not been reached in this research field. Thus, we only applied SNPSpD correction for genotypic and allelic association analysis for four SNPs, because the number of effective independent SNPs could be calculated by the SNPSpD method.

The reason why we obtained such low p values in our association analysis could be due to a relatively large sample size and high frequency of minor allele of the SNP4. Indeed, power analysis showed that our subjects had sufficient power (>0.95) to detect an effect of the odds ratios for SNP4, 1.23 in total subjects and 1.32 in male subjects. Although the strong association between the *RELA* gene and schizophrenia has been observed, the biological significance of this gene in susceptibility for schizophrenia might not be large, because 22% of the patients with schizophrenia have homozygous of risk allele in SNP4, but 16% of the controls also are homozygous of risk allele in SNP4. However, the association between the *RELA* gene and schizophrenia
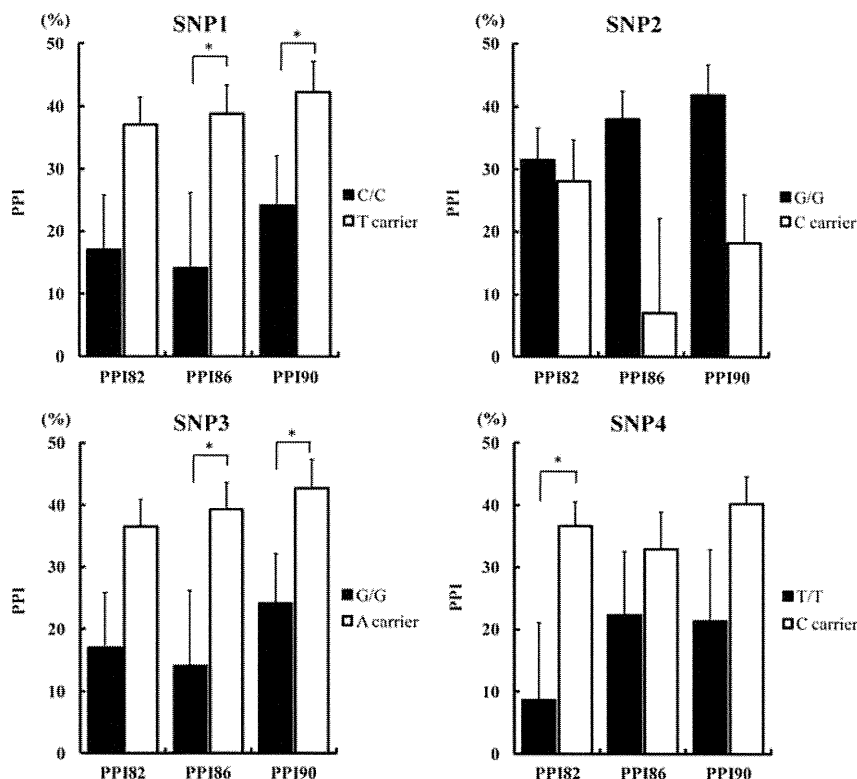
Figure 2 Effects of the genotypes in the RELA gene on PPI. PPI82, PPI86, and PPI90: pre-pulse inhibition of acoustic startle reflex in pre-pulse intensities of 82 dB, 86 dB, and 90 dB, respectively. Error bars represent the standard error of the mean. *$p<0.05$. **$p<0.01$.

might explain, at least in part, the relation between immune system and schizophrenia.

It is of interest to study how genetic variation affects *RELA* function/expression. There is no experimental-based evidence that any of the SNPs or haplotypes is functional. Very little is known about the potential function of specific intronic sequences with regard to protein binding, stability, and splicing efficacy. However, the genotype-expression analysis showed that the risk alleles are associated with lower *RELA* gene expression in Japanese lymphoblasts. On the other hand, no association was found between other SNPs and gene expression in other ethnicities. No data could explain these results. A possible explanation for this ethnic difference is that the allele frequencies of some target genes in the signal transduction pathway of NF-κB, such as interleukins, chemokines, and interferons, and molecules involved in apoptosis or adhesion (Li and Verma, 2002), which could be associated with the pathophysiology of schizophrenia, might be different among populations. For example, a GWAS study in Caucasian population showed significant association with schizophrenia were in a region of LD on chromosome 6p22.1, including several immunity related genes other than the *RELA* gene (Stefansson *et al*, 2009). There were four SNPs associated with schizophrenia in the immunity related genes. Three SNPs out of four SNPs were not polymorphic in Japanese population and the minor allele frequency of the remained one SNP is 0.01 in Japanese population (0.19 in Caucasian population). This difference of allele frequency of SNP could alter the effect of the presumably functional risk

alleles in the *RELA* gene in other ethnicities. A risk SNP of the *RELA* gene was found in the possible transcription factor binding sequence of AR. Indeed, it was reported that AR activation decreased the expression of *RELA* and reduced its nuclear localization and transcriptional activity (Nelius *et al*, 2007). As the risk allele destroyed the consensus sequence for AR binding, this SNP could be functional. Several studies have examined the association between AR and schizophrenia, and there are clinical differences between males and females, such as greater lifetime risk, earlier age of onset and poorer outcome in males (Tandon *et al*, 2008). Crow *et al*. reported the association of AR with schizophrenia in males (Crow *et al*, 1993); however, negative results have also been reported (Arranz *et al*, 1995; Tsai *et al*, 2006). When we examined the association between the *RELA* gene and schizophrenia by gender, a significant association was observed in males but not in females. Our results suggest that the T allele of SNP4 in the *RELA* gene, which might be functional for AR binding and transcription, could be a risk-associated allele for male schizophrenia.

There are numerous genes in neurons that are regulated by NF-κB (Kaltschmidt *et al*, 2005). Among these genes are molecules related to neurotransmission, including subunits of N-methyl-D-aspartate receptors, voltage-dependent calcium channels and the calcium-binding protein calbindin; cell survival factors, including Bcl-2, Mn-SOD, and inhibitor of apoptosis proteins (IAPs); and cell death factors, including Bcl-x(S) and Bax (Mattson, 2005). It is noteworthy that the expression levels of some of these downstream

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molecules have been reported to be altered in postmortem brains of patients with schizophrenia, for example, increased: Bax, calbindin, and NR2B; decreased Bcl-2 (Gao et al, 2000; Jarskog et al, 2000, 2004; Fung et al, 2010).

PPI of the acoustic startle response has been demonstrated from mice to humans, and is considered to be a measure of 'sensorimotor gating,' whereby pre-pulses reduce the effect of subsequent sensory stimuli to protect the brain from sensory overload (Braff and Geyer, 1990). A deficit in PPI is a reliable feature of schizophrenia, where reduced gating is thought to be one possible neurobiological mechanism that underlies the basic cognitive abnormalities associated with this disorder (Braff et al, 2001). We observed associations between risk SNPs in the *RELA* gene and some pre-pulse intensities in patients with schizophrenia. Although we cannot explain the differences in the associated pre-pulse intensities among SNPs, our findings suggest that the *RELA* gene might modulate PPI in patients with schizophrenia.

The PPI deficits observed in schizophrenia can be mimicked in animals by the administration of dopamine agonizts and NMDA antagonists, such as phencyclidine (PCP), and reversed by anti-psychotic drugs (Mansbach et al, 1988; Geyer et al, 2001; Wang et al, 2001). Typical and atypical anti-psychotic drugs also reverse the PPI deficits observed in schizophrenic patients (Kumari and Sharma, 2002). Consistent with our data, PCP administration to rats that showed deficits in PPI elicited the abnormal nuclear translocation of NF-κB in the frontal cortex (Wang et al, 2001), which is indicative of a functional correlation between the *RELA* gene and PPI in a potential animal model for schizophrenia.

There are several limitations to interpret our results. Our study size of 1224 cases and 1663 controls had sufficient power (>0.80) to detect the effects of odds ratios of 1.16 or greater as indicated in recent genome-wide association studies for each SNP (O'Donovan et al, 2009). However, the possibility of false positive results due to type I errors could not be excluded. Our positive results might be derived from sample bias due to population stratification and non-sex-matched samples, although the Japanese are a relatively homogeneous population and the logistic regression study revealed genotype effects on the diagnosis independent of sex. We did not perform a systematic mutation search using these Japanese schizophrenia samples. In silico analysis of gene expression in lymphoblasts and AR binding site in SNP4, raised a possibility that the SNP4 might be a functional SNP. However, further biological study of the function of SNP4 is required to verify these in silico results. As the sample size we used for the PPI analysis was small, gender effect on the association between PPI and the SNPs in the *RELA* gene was not able to be analyzed. An increased sample size for schizophrenia and control subjects is needed before a firm conclusion can be drawn. We used PPI as a main phenotype of interest. Other phenotypes such as neurocognitive dysfunction that has larger effect size than PPI and brain morphology, which is more stable overtime were not tested in this study; however, PPI is a physiological phenotype, which is reliable, easily measured, and relatively specific for schizophrenia. In the present study, we propose *RELA* as a new candidate gene for susceptibility to schizophrenia.

DISCLOSURE

The authors RH, KO, YY, MF, HY, HT, MI, TO, HK, OS, MT, NI, NO, KK, HK and MT 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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ORIGINAL ARTICLE

Dysbindin-1 and NRG-1 gene expression in immortalized lymphocytes from patients with schizophrenia

Hidenaga Yamamori[1,2], Ryota Hashimoto[2,3], Louise Verrall[2], Yuka Yasuda[2], Kazutaka Ohi[2], Motoyuki Fukumoto[2], Satomi Umeda-Yano[1], Akira Ito[1] and Masatoshi Takeda[2]

The dysbindin-1 and neuregulin-1 (NRG-1) genes are related to schizophrenia. Expression studies in postmortem brains have revealed lower expression of dysbindin-1 and higher expression of NRG-1 in brain tissue from subjects with schizophrenia. In addition to the difficulty of sampling, the use of postmortem brain tissues is not ideal because these tissues are heterogeneous with respect to biochemical parameters, lifetime history of medications and physiological status at the time of death. In contrast, medication and environmental influences that could mask the genetic basis of differences in RNA expression are removed in immortalized lymphocytes by culturing. Only a few microarray analysis studies using immortalized lymphocytes in schizophrenia have been reported, and whether immortalized lymphocytes are an appropriate alternative to neuronal tissue remains controversial. In this study, we measured the mRNA expression levels of dysbindin-1, NRG-1 and two other genes (NPY1R and GNAO1) in immortalized lymphocytes from 45 patients with schizophrenia and 45 controls using real-time quantitative reverse transcriptase-PCR. No difference was observed between patients and controls with respect to the expression of dysbindin-1, NRG-1, NPY1R or GNAO1 gene. Our findings suggest that the gene expression profile of immortalized lymphocyte from schizophrenic patients is different from that in postmortem brain tissue at least with respect to the dysbindin-1 and NRG-1 genes.

INTRODUCTION

Schizophrenia is a complex genetic disorder that is characterized by profound disturbances of cognition, emotion and social functioning. It affects ~1% of the general population world wide. The dysbindin-1 and neuregulin-1 (NRG-1) genes are related to schizophrenia,[1] and the dysbindin-1 gene is also associated with cognitive functions.[2–4] Furthermore, the Sandy mouse, which expresses no dysbindin-1, has been reported to have behavioral abnormalities, cognitive deficits and a synaptic dysfunction that is related to the pathophysiology of schizophrenia.[5–7] Identified risk variants of NRG-1 are associated with the reduced white matter volume that is observed in schizophrenic brains.[8] The NRG-1 gene spans 1.2 Mb[9] and gives rise to many structurally and functionally distinct isoforms, through alternative promoter usage. These isoforms are divided into three classic groups:[10] type I (previously known as acetylcholine receptor inducing activity, heregulin or neu differentiation factor), type II (glia growth factor) and type III (cysteine-rich domain containing), which are based on distinct amino termini. Additional NRG-1 5′ exons have

recently been identified, giving rise putatively to novel NRG-1 types IV–VI in the human brain.[11] Transgenic mice that overexpress NRG-1 type I, the expression of which is reported to be increased in the schizophrenic brains,[12] have a tremor, show impaired ability on the accelerating rotarod and have reduced prepulse inhibition.[13] Expression studies in postmortem brains have also revealed lower expression of dysbindin-1 and higher expression of NRG-1 type I, in subjects with schizophrenia.[12,14–16]

Postmortem brain tissues are necessary for determining the pathophysiology of schizophrenia. Many gene expression studies have been conducted using postmortem brain tissues. These studies have demonstrated increased expression of genes involved in pre-synaptic function[17,18] and the downregulation of myelination-related genes.[19,20] Although there is some agreement across these studies, there has been a lack of consistency because of the varying characteristics of postmortem brain tissues. Postmortem brain tissues are not easy to obtain. Moreover, postmortem brain tissues are quite heterogeneous with respect to biochemical parameters, lifetime history of

[1]Department of Molecular Neuropsychiatry, Osaka University Graduate School of Medicine, Osaka, Japan; [2]Department of Psychiatry, Osaka University Graduate School of Medicine, Osaka, Japan; and [3]Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, Osaka, Japan
Correspondence: Dr R Hashimoto, Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, D3, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan.
E-mail: hashimor@psy.med.osaka-u.ac.jp

medications and physiological status at the time of death and sampling.

In contrast, medication and environmental influences that could mask the genetic basis of differences in RNA expression may be removed in immortalized lymphocytes by culturing. Immortalized lymphocytes can be readily obtained, in contrast to postmortem brain tissues, thereby allowing larger case–control expression studies with optimal matching on key variables such as age and sex. The gene expression profile of whole blood has been shown to have moderate degree of similarity to that of the central nervous system among 79 human tissues.[21] For these reasons, immortalized lymphocytes are good tools for determining the effect of genetic risks or drug treatment on gene expressions. Disrupted in schizophrenia 1 and a serotonin transporter polymorphism have been reported to have effect on their gene expressions in immortalized lymphocytes from bipolar disorder patients.[22,23] The effect of lithium on gene expression was also investigated in immortalized lymphocytes from bipolar disorder patients.[24] However, only a few microarray analysis using immortalized lymphocytes from patients with schizophrenia have been reported.[25–27] Whether immortalized lymphocytes are an appropriate alternative to neuronal tissue remains controversial.

A recent study using microarray analysis has shown that the expression levels of *dysbindin-1* isoform A and the *NRG-1* type II GGF2 isoform in immortalized lymphocytes are lower in patients with schizophrenia than in controls. In contrast, the expression of the *NRG-1* type II GGF isoform was not significantly different between patients with schizophrenia and controls.[28] In this study, the expression profiles of *dysbindin-1* and *NRG-1* in immortalized lymphocytes were partly consistent with those in postmortem brains. However, a limited number of subjects were used, and a limited number of *dysbindin-1* and *NRG-1* isoforms was observed, therefore whether the expression profile of *dysbindin-1* and *NRG-1* in immortalized lymphocytes is consistent with that in the postmortem brain remains controversial.

In this study, we used approximately four times as many subjects as the previous study. We observed the total expression of *dysbindin-1* and *NRG-1* (that is, the combined expression of all isoforms), which had previously been observed in a postmortem brain,[12,14–16] to determine whether immortalized lymphocytes are a good tool to determine the effect of genetic risks of *dysbindin-1* and *NRG-1* on their expression and whether immortalized lymphocytes are an appropriate alternative to neuronal tissue.

MATERIALS AND METHODS

Subjects

In all, 45 Japanese patients with schizophrenia and 45 healthy Japanese control subjects participated in this study. Patients were recruited at Osaka University Hospital. Controls were recruited by local advertisements in Osaka.

Consensus diagnosis was made for each patient by at least two trained psychiatrists, according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, criteria using the structured clinical interview for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (severe combined immunodeficiency). Controls were psychiatrically, medically and neurologically healthy volunteers who were not receiving psychiatric medication and had no first- or second-degree relatives with psychoses. Controls were screened for psychiatric disease with the non-patient edition of the modified structured clinical interview for the Diagnostic and Statistical Manual, Fourth Edition, Axis I disorders (severe combined immunodeficiency-I/non-patient). Symptoms of schizophrenia were assessed using the positive and negative syndrome scale. The clinical and demographic characteristics of all subjects are presented in Table 1. Patients and controls were matched for age and sex. All patients were treated with antipsychotics.

Immortalized lymphocytes and RNA extraction

The isolation of lymphocytes from the blood and immortalization using Epstein–Bar virus were performed by the Special Reference Laboratories (Tokyo, Japan). Immortalized lymphocytes from 45 patients with schizophrenia and from 45 controls were grown in culture media supplemented with 20% fetal bovine serum. Total RNA was extracted from cell pellets using the RNeasy mini kit (QIAGEN KK, Tokyo, Japan). The yield of total RNA was determined by measuring the absorbance at 260 nm, and the quality of the total RNA was analyzed using agarose gel electrophoresis.

DNase treatment and reverse transcription

Total RNA was treated with DNase to remove contaminating genomic DNA using the DNase Treatment & Removal Reagents (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Total RNA (10 µg) treated with DNase was used in a 50 µl reverse transcriptase (RT) reaction to synthesize complementary DNA using the SuperScript first-strand synthesis system for reverse transcriptase-PCR (RT-PCR; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly, total RNA (10 µg) was denatured in the presence of 1 mM deoxyribonucleotide triphosphates and 5 ng µl^{-1} random hexamers at 65 °C for 5 min. After the addition of RT buffer, MgCl$_2$ (5 mM final concentration), dithiothreitol (10 mM final concentration), RNAseOUT recombinant ribonuclease inhibitor (100 U) and SuperScriptIII RT (125 U), the reaction mixture was incubated at 25 °C for 10 min, at 42 °C for 40 min and at 70 °C for 15 min. RNase H (5 U) was added to the reaction mixture, and then the mixture was incubated at 37 °C for 20 min to stop the reaction.

Table 1 The clinical and demographic characteristics of all subjects

| | Patients with schizophrenia | Healthy controls | Group difference | | |
			Z	d.f.	P-value
N	45	45			
Age (year)	37.9 ± 11.0	38.1 ± 11.3	−0.194		0.846
Sex (%M; male/female)	57.8% (26/19)	57.8% (26/19)		1	1[a]
Age of onset (year)	24.2 ± 9.8	NA			
Duration of illness (year)	13.7 ± 10.1	NA			
CPZeq dose (mg per day)	587.4 ± 522.4	NA			
PANSS positive	18.5 ± 6.8	NA			
PANSS negative	19.2 ± 6.8	NA			
PANSS general psychopathology	38.0 ± 10.3	NA			

Abbreviations: M, male; NA, not available; PANSS, positive and negative syndrome scale.
Means ± s.d. are shown.
[a]χ^2-test was used.

Oligonucleotide and primer design

The TaqMan Pre-Developed Assay Reagent kit (Applied Biosystems, Foster City, CA, USA) was used for the analysis of two housekeeping genes, *β-actin* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and for *dysbindin-1* (Hs00225229_m1), *NPY1R* (Hs00702150_s1) and *GNAO1* (Hs00221365_m1).

The primer and probes for *NRG-1* types I to IV were as described previously.[12,16] The real-time PCR (TaqMan) detection of *NRG-1* isoforms used the following oligonucleotides: type I, forward primer 5′-GCCAATAT CACCATCGTGGAA-3′, reverse primer 5′-CCTTCAGTTGAGGCTGGCATA-3′, probe 5′-FAM-CAAACGAGATCATCACTGMGB-3′; type II, forward primer 5′-GAATCAAACGCTACATCTACATCCA-3′, reverse primer 5′-CCTTCTCCG CACATTTTACAAGA-3′, probe 5′-FAM-CACTGGGACAAGCC-MGB-3′; type III, forward primer 5′-CAGCCACAAACAACAGAAACTAATC-3′, reverse primer 5′-CCCAGTGGTGGATGTAGATGTAGA-3′, probe 5′-FAMCCAAAC TGCTCCTAAAC-MGB-3′ and type IV, forward primer 5′-GCTCCGGCAGC AGCAT-3′, reverse primer 5′-GAACCTGCAGCCGATTCCT-3′, probe 5′-FAM-ACCACAGCCTTGCCT-MGB-3′ (purchased from Applied Biosystems). These primers were designed to amplify specific transcripts based on the unique exon structure of each isoform. Thus, for example, because isoform II lacks exons 5–7, primers complementary to sections of exons 4 and 8, which are contiguous in the isoform II transcript, will only amplify this isoform.

Real-time quantitative RT-PCR

Dysbindin-1 and *NRG-1* mRNA expression levels were measured by real-time quantitative RT-PCR, using each combination of oligonucleotides and an ABI Prism 7900 sequence detection system with a 384-well format (Applied Biosystems). Each 20 μl PCR reaction contained 6 μl of complementary DNA, 900 nM of each primer, 250 nM of probe and 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase UNG, deoxyribonucleotide triphosphates with deoxyuridine triphosphate, a passive reference and optimized buffer components. The PCR cycling conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 59 °C or 60 °C for 1 min. PCR data were obtained using the Sequence Detector Software (version 2.1, Applied Biosystems) and were quantified by

a standard curve method. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at the threshold (C_t; where the fluorescence generated within a reaction and threshold cross) versus the quantity of RNA. All samples were measured in one plate for one target gene or isoform, and their C_t-values were in the linear range of the standard curve. Experiments were typically performed three times with triplicate determinations and the expression level of each gene was determined by the average of the three independent experiments. Predicted C_t-values and sample quantities were used for statistical analysis.

Statistical analysis

The individual Mann–Whitney U-test and the χ^2-test were used to compare means and categorical proportions, respectively. The groups did not differ with respect to age or gender (Table 1). The differences in the mRNA levels of *dysbindin-1*, *NRG-1* type II, *NPY1R* and *GNAO1* between patients and controls were also analyzed by analysis of covariance, with diagnosis as the independent factor and sex and age as covariates. Spearman rank order correlation test was performed to assess the possible correlation between gene expressions and clinical characteristics.

RESULTS

To measure the expression levels of *dysbindin-1* and *NRG-1* in immortalized lymphocytes, standard curves were obtained using serial dilutions (1:4) of pooled complementary DNA prepared from 300 ng total RNA derived from immortalized lymphocytes. For *NRG-1* types I, II, III and IV, the same amount of RNA from a postmortem brain (a kind gift from the Stanley Foundation) was also used as a positive control. The standard curves of two housekeeping genes, *β-actin* and *GAPDH*, and of *dysbindin-1* showed that these genes were expressed in immortalized lymphocytes (Figures 1a–c). Although the expression of *NRG-1* type II was observed in immortalized lymphocytes, the expression levels of *NRG-1* types I, III and IV
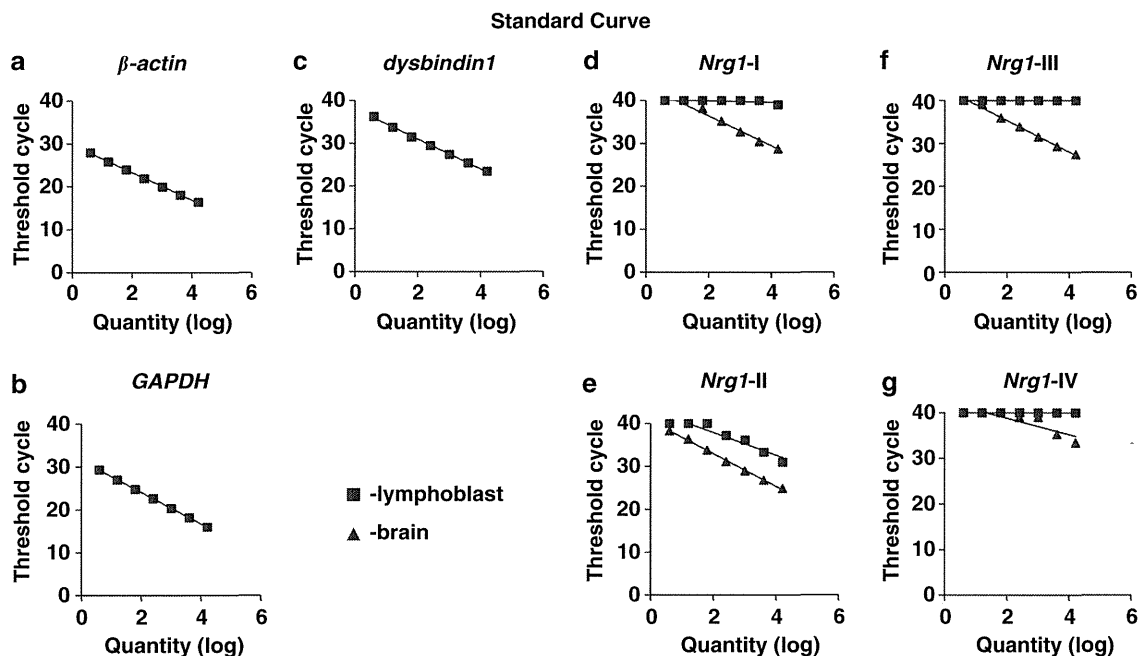
Standard Curve

Figure 1 Standard curves for the housekeeping genes, *dysbindin-1* and the four *NRG-1* isoforms. (**a, b**) Standard curves for the house keeping genes, *β-actin* (a) and *GAPDH* (b). (**c**) Standard curve for *dysbindin-1*. The expression of the *dysbindin-1* gene was observed in immortalized lymphocytes. (**d–g**) Standard curves for *NRG1* types I (**d**), II (**e**), III (**f**) and IV (**g**). The expression of *NRG-1* types I, II, III and IV was observed in the postmortem brain, as previously reported. The expression of *NRG-1* type II was observed in immortalized lymphocytes. The expression levels of *NRG-1* types I, III and IV in immortalized lymphocytes were below the detection limit of the real-time quantitative RT-PCR assay.

were below the detection limit of the real-time quantitative RT-PCR assay (Figures 1d–g). The expressions of *NRG-1* types I, II, III and IV in the postmortem brain were confirmed by the real-time quantitative RT-PCR method, as previously reported (Figures 1d–g).[12,16] In each experiment for *β-actin*, *GAPDH*, *dysbindin-1* and *NRG-1* type II the R^2-value of the standard curve was >0.99, and no-template control assays resulted in no detectable signal.

The expression levels of *β-actin* and *GAPDH* were not significantly different between the 45 patients with schizophrenia and the 45 age- and sex-matched controls (Figure 2a). No significant difference was observed between the 45 patients with schizophrenia and the 45 controls with respect to the expression of *dysbindin-1* and *NRG-1* type II normalized to the expression of *β-actin* or *GAPDH* (Figures 2b and c; Mann–Whitney *U*-test: *dysbindin-1/β-actin*; $U=916$, $P=0.436$, *dysbindin-1/GAPDH*; $U=952$, $P=0.625$, *NRG-1* type II/*β-actin*; $U=961$, $P=0.678$, *NRG-1* type II/*GAPDH*; $U=977$, $P=0.775$). Analysis of covariance with sex and age as covariates did not alter the results (*dysbindin-1/β-actin*; $F=0.267$, $P=0.607$, *dysbindin-1/GAPDH*; $F=0.06$, $P=0.808$, *NRG-1* type II/*β-actin*; $F=2.412$, $P=0.124$, *NRG-1* type II/*GAPDH*; $F=1.693$, $P=0.197$).

A correlation test was performed to observe the influence of clinical characteristics on the expression levels of *dysbindin-1* and *NRG-1* type II in immortalized lymphocytes from patients with schizophrenia. None of the measurements of *dysbindin-1* and *NRG-1* type II expression normalized by *β-actin* and *GAPDH* expression correlated significantly with age, age of onset, duration of illness, chlorpromazine equivalents, positive and negative syndrome scale positive, negative or general psychopathology scores (Spearman rank order correlation test: all $P>0.1$).

The expression levels of *NPY1R* and *GNAO1*, which were reported to be differentially expressed in schizophrenic immortalized lymphocytes,[25] were also measured to further assess the usefulness of immortalized lymphocytes from patients with schizophrenia. No significant difference was 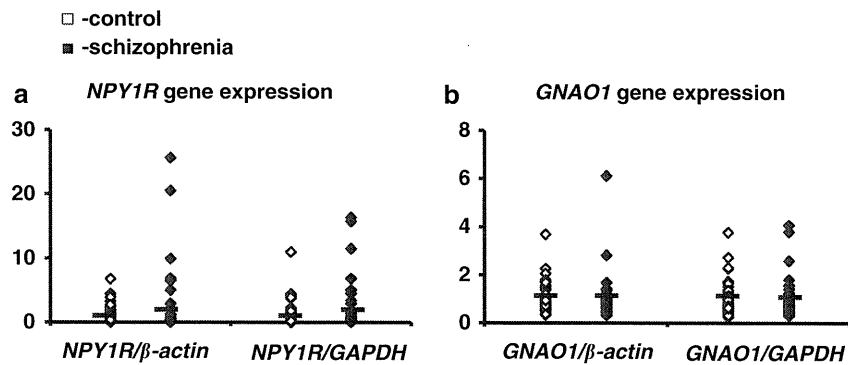observed between the 45 patients with schizophrenia and the 45 controls with respect to the expression of *NPY1R* and *GNAO1* normalized to the expression of *β-actin* or *GAPDH* (Figures 3a and b) (Mann–Whitney *U*-test: *NPY1R/β-actin*; $U=949$, $P=0.606$, *NPY1R/GAPDH*; $U=949$, $P=0.608$, *GNAO1/β-actin*; $U=932$, $P=0.516$, *GNAO1/GAPDH*; $U=965$, $P=0.701$). An analysis of covariance with sex and age as covariates did not alter the results (*NPY1R/β-actin*; $F=2.940$, $P=0.090$, *NPY1R/GAPDH*; $F=1.756$, $P=0.189$, GNAO1/β-actin; $F=0.004$, $P=0.950$, *GNAO1/GAPDH*; $F=0.007$, $P=0.935$).

DISCUSSION

We confirmed the expression of *dysbindin-1* and *NRG-1* types I, II, III and IV in postmortem brain.[16] However, the expression levels of *NRG-1* types I, III and IV were below the detection limit of the real-time quantitative RT-PCR assay, and only *dysbindin-1* and *NRG-1* type II expression was observed in immortalized lymphocytes. In the postmortem brain, the expression of *dysbindin-1* has been reported to be lower in patients with schizophrenia than in controls.[14,15] *NRG-1* type I in the postmortem brain has been reported to be higher in patients with schizophrenia than in controls, and the expression of *NRG-1* types II, III and IV in the postmortem brain has been reported to show no significant difference between patients with schizophrenia and controls.[12,16] In immortalized lymphocytes, we found no difference between patients with schizophrenia and controls with respect to the expression of *dysbindin-1* and *NRG-1* type II. The expression profile of *NRG-1* type II in immortalized lymphocytes was consistent with that in the postmortem brain, but the expression profile of *dysbindin-1* was not consistent with that in the postmortem brain. Our findings suggest that, in subjects with schizophrenia, the immortalized lymphocyte gene expression profile is different from that in postmortem brain tissue at least with respect to *dysbindin-1* and *NRG-1* genes. This difference in gene expression profile might be attributed to the differences of the tissue-specific regulation of gene expression and alternative splicing. Not only the tissue-specific regulation
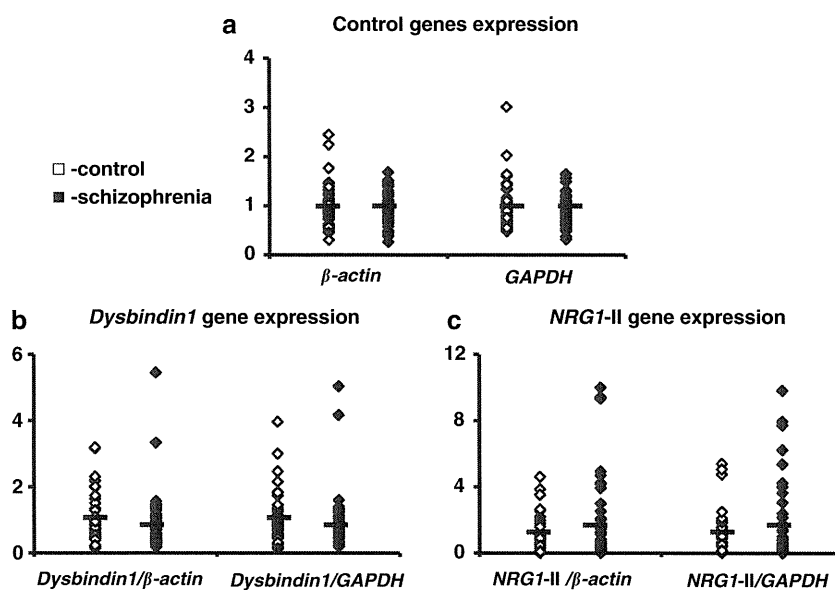
Figure 2 Control gene, *dysbindin-1* and *NRG-1* type II expression levels. (a) The expression levels of the house keeping genes *β-actin* and *GAPDH*. The expression levels of the two standard housekeeping genes were not significantly different between patients with schizophrenia and controls. (b) The expression levels of *dysbindin-1* normalized by *β-actin* and *GAPDH* expression. (c) The expression levels of *NRG-1* type II normalized by *β-actin* and *GAPDH* expression. No significant difference was observed between the 45 patients with schizophrenia and the 45 controls with respect to the expression of *dysbindin-1* and *NRG-1* type II normalized by *β-actin* and *GAPDH* and expression. The bars show the means.

Figure 3 *NPY1R* and *GNAO1* expression levels. (a) The expression levels of *NPY1R* normalized by *β-actin* and *GAPDH* expression. (b) The expression levels of *GNAO1* normalized by *β-actin* and *GAPDH* expression. No significant difference was observed between the 45 patients with schizophrenia and the 45 controls with respect to the expression of *NPY1R* and *GNAO1* normalized by *β-actin* and *GAPDH* expression. The bars show the means.

of gene expression but also the transformation process using Epstein–Bar virus and culturing might have effect on this difference in gene expression profiles. The expression profile in postmortem brain might be affected by medication and environmental influences that could be removed in immortalized lymphocytes by this transformation and culturing process.

We failed to replicate the abnormal expression of *dysbindin-1* and *NRG-1* in immortalized lymphocytes in our cohort, which consist of a much larger sample than that used in the previous study. The previous study showed that *dysbindin-1* isoform A and *NRG-1* type II isoform GGF2 in immortalized lymphocytes from patients with schizophrenia were decreased relative to that in controls.[28] This discrepancy might be attributed to the differences in the isoforms observed, the sample size, the ethnicity of the subjects and the sample preparation. In this study, we used approximately four times more subjects than the previous study, and we observed the total expression of the genes (that is, the combined expression of all isoforms) in a Japanese sample population. To clarify this discrepancy, we should observe the expression levels of all of the isoforms of the genes individually (that is, *dysbindin-1* isoform A and *NRG-1* type II isoform GGF).

We have also measured the mRNA expression levels of *NPY1R* and *GNAO1*, which were reported to be differentially expressed in schizophrenic immortalized lymphocytes,[25] using our cohort which consist of larger sample to further assess the usefulness of immortalized lymphocytes from patients with schizophrenia. We also failed to replicate the abnormal expression of *NPY1R* and *GNAO1* in immortalized lymphocytes in our cohort, which consist of a much larger sample than that used in the previous study. This discrepancy might be attributed to the differences of the sample size, the ethnicity of the subjects or the sample preparation.

Although the *dysbindin-1* and *NRG-1* gene expression profiles in immortalized lymphocytes were different from those in postmortem brain tissue, it remains possible that immortalized lymphocytes could be good tools to determine the effect of genetic risks of the *dysbindin-1* and *NRG-1* genes on their expression, for example, the allele effects that have been reported to be associated with schizophrenia on their genes expressions. In immortalized lymphocytes, it might be difficult to observe the effect of *dysbindin-1* and *NRG-1* gene expression on their neuron-specific functions, for example, the effect of *dysbindin-1* on glutamate and dopamine release,[5,6,29] and on the formation of synaptic vesicles[30] and the effect of *NRG-1* on N-methyl D-aspartate receptor hypofunction.[31] However, we might be able to determine the effect of *dysbindin-1* and *NRG-1* genes expression on their functions

which are common in multiple tissues using immortalized lymphocytes, for example, the effect of *dysbindin-1* on phosphatidylinositol 3 kinase–Akt signaling[29] and the effect of *NRG-1* on ErbB–Akt signaling. In fact, it has been reported that *NRG-1*-induced cell migration resulting from ErbB–Akt signaling is impaired in immortalized lymphocyte from patients with schizophrenia.[32]

Further studies are required to assess whether immortalized lymphocytes are a good tool to determine the effect of genetic risks on their gene expression and whether immortalized lymphocytes are an appropriate alternative to neuronal tissue.

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*Molecular
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Gene expression analysis in lymphoblasts derived from patients with autism spectrum disorder

Yuka Yasuda[1,2], Ryota Hashimoto[1,2,3*], Hidenaga Yamamori[1,4], Kazutaka Ohi[1,2], Motoyuki Fukumoto[1,2], Satomi Umeda-Yano[4], Ikuko Mohri[3,5], Akira Ito[4], Masako Taniike[3,5] and Masatoshi Takeda[1,3]

Abstract

Background: The autism spectrum disorders (ASDs) are complex neurodevelopmental disorders that result in severe and pervasive impairment in the development of reciprocal social interaction and verbal and nonverbal communication skills. In addition, individuals with ASD have stereotypical behavior, interests and activities. Rare mutations of some genes, such as neuroligin (*NLGN*) 3/4, neurexin (*NRXN*) 1, *SHANK3*, *MeCP2* and *NHE9*, have been reported to be associated with ASD. In the present study, we investigated whether alterations in mRNA expression levels of these genes could be found in lymphoblastoid cell lines derived from patients with ASD.

Methods: We measured mRNA expression levels of *NLGN3/4*, *NRXN1*, *SHANK3*, *MeCP2*, *NHE9* and *AKT1* in lymphoblastoid cells from 35 patients with ASD and 35 healthy controls, as well as from 45 patients with schizophrenia and 45 healthy controls, using real-time quantitative reverse transcriptase polymerase chain reaction assays.

Results: The mRNA expression levels of *NLGN3* and *SHANK3* normalized by *β-actin* or *TBP* were significantly decreased in the individuals with ASD compared to controls, whereas no difference was found in the mRNA expression level of *MeCP2*, *NHE9* or *AKT1*. However, normalized *NLGN3* and *SHANK3* gene expression levels were not altered in patients with schizophrenia, and expression levels of *NLGN4* and *NRXN1* mRNA were not quantitatively measurable in lymphoblastoid cells.

Conclusions: Our results provide evidence that the *NLGN3* and *SHANK3* genes may be differentially expressed in lymphoblastoid cell lines from individuals with ASD compared to those from controls. These findings suggest the possibility that decreased mRNA expression levels of these genes might be involved in the pathophysiology of ASD in a substantial population of ASD patients.

Background

Autism spectrum disorder (ASD), also known as pervasive developmental disorder (PDD), is defined as severe and pervasive impairments in the development of reciprocal social interaction and verbal and nonverbal communication skills. These disorders are also characterized by stereotypical behavior, interests and activities. The lifetime morbidity rate of ASD is 0.2% to 1.0% across studies [1]. In addition, twin and family studies of ASD have demonstrated a high heritability of approximately 90% [2], indicating that ASD is a heterogeneous condition that is likely to result from the combined effects of

multiple genetic factors interacting with environmental factors. Recent genetic studies have identified several vulnerability loci and genetic mutations that cause ASD. One of the most striking revelations is the important role of genes that encode proteins at the neuronal synapse [3].

Rare mutations in the neuroligin 3 (*NLGN3*) and neuroligin 4 (*NLGN4*) genes, which map to chromosomes Xq13 and Xp22.3, have been reported in some patients with ASD and other neurodevelopmental impairments [4-8]. A particular mutation of *NLGN3* (Arg451Cys) is known to cause a defect in protein processing of *NLGN3* [9]. In addition, a particular mutation of *NLGN4* (1186insT) causes a frameshift mutation that leads to premature termination of *NLGN4* (D396X), resulting in a loss of 421 amino acids (51% of the

* Correspondence: hashimor@psy.med.osaka-u.ac.jp
[1]Department of Psychiatry, Osaka University Graduate School of Medicine, D3, 2-2, Yamadaoka, Suita, 565-0871, Osaka, Japan
Full list of author information is available at the end of the article

protein) [4]. Neuroligins, which are postsynaptically localized cell adhesion molecules, play a crucial role in organizing excitatory glutamatergic and inhibitory GABAergic synapses in the mammalian brain by interacting with presynaptic β-neurexins (*NRXN*), thereby triggering the formation of functional presynaptic structures in contacting axons [10]. Mutations of the neurexin 1 (*NRXN1*) gene, at the chromosome locus 2q32, have been found in individuals with ASD [11-14]. Furthermore, *de novo* copy number variation analysis revealed deletion of the *NRXN1*-containing gene region in ASD [15]. The binding of *NRXN1* and *NLGN* genes mediates synaptic development [16]. Interestingly, a mutation of *NLGN3* results in a disruption of the ability to bind to *NRXN* [9]. In addition, neuroligins interact with a postsynaptic scaffolding protein, SHANK3, which is also implicated in ASD [17] and is located on the telomeric terminal of chromosome 22q13.3. Shank proteins couple neurotransmitter receptors, ion channels and other membrane proteins to the actin cytoskeleton and G protein-coupled signaling pathways, and they also play a role in synapse formation and dendritic spine maturation [18]. Deletion or translocation of the genomic locus, which includes the *SHANK3* gene, and *de novo* mutations of the *SHANK3* gene result in premature stop codons and have been found in ASD [17,19,20].

In a study of consanguineous autism families, Morrow *et al.* [21] observed a relationship between ASD and alterations in the sodium/hydrogen exchanger 9 (*NHE9*) gene. Specifically, they found a nonsense mutation in patients with ASD that is a heterozygous CGA-to-TGA transition, changing arginine 423 to a stop codon [21]. The *NHE9* gene is located on chromosome 3q24 and is one of the families of Na^+/H^+ exchangers that regulate ion flux across membranes [22]. Rett syndrome is another PDD, and the methyl-CpG-binding protein 2 (*MeCP2*) gene is a causal gene for Rett syndrome. *MeCP2* is a transcriptional repressor that binds to methylated CpG dinucleotides generally located at gene promoters and recruits histone deacetylase 1 and other proteins involved in chromatin repression [23]. *De novo* mutations of the *MeCP2* gene located on chromosome Xp28 occur in 80% of female patients with Rett syndrome [24]. Some evidence of dysregulation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway is implicated in ASD, despite the fact that no mutation which causes ASD has been reported in association with the *AKT1* gene. The expression and phosphorylation and/or activation of AKT were found to be decreased in the autistic brain [25]. The *PTEN* gene (phosphatase and tensin homolog deleted on chromosome 10) is a major negative regulator of the PI3K/AKT pathway, and *PTEN* mutations have been linked to ASD [26].

Recently, several studies have suggested that lymphoblastoid cells can be used to detect biologically plausible correlations between candidate genes and neuropsychiatric diseases, including Rett syndrome [27], nonspecific X-linked mental retardation [28], bipolar disorder [29], fragile X syndrome [30,31] and dup(15q) [32]. In the present study, we compared mRNA expression levels of various genes in blood-derived lymphoblastoid cells from individuals with ASD and healthy controls.

Methods
Participants
We obtained mRNA samples from patients with ASD, patients with schizophrenia and healthy controls from the research bioresource of the Human Brain Phenotype Consortium in Japan (http://www.sp-web.sakura.ne.jp/consortium.html). The ASD cohort consisted of 35 patients with ASD and healthy controls (Table 1). Patients with ASD and patients with schizophrenia were recruited from both outpatient and inpatient services at Osaka University Hospital. Each ASD patient was diagnosed by at least two trained child psychiatrists and/or child neurologists according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition-Text Revision* (DSM-IV-TR) criteria based on unstructured or semistructured behavioral observations of the patients and interviews with the patients and their parents or caregivers. During the interview, the Pervasive Developmental Disorders Autism Society Japan Rating Scale (PARS) [33] and the Japanese version of the Asperger's Questionnaire [34] were used to assist in the evaluation of ASD-specific behaviors and symptoms. PARS is a semistructured interview that is composed of

Table 1 Demographic information for the ASD and control cohorts[a]

Demographics	ASD (n = 35)	Controls (n = 35)	P value
Sex, M/F	27/8	26/9	$\chi^2 = 0.078$ (1, $N = 70$), $P = 0.78$
Mean age, years (± SD)	12.9 (12.4)	34.8 (9.7)	$U = 86$, $P = 0.60 \times 10^{-9}$, $Z = -6.19$
Age range, years	3 to 63	21 to 65	
Number of ASD (with IQ < 70)	35 (11)	0	
Number of Autism (with IQ < 70)	20 (10)	-	
Number of Asperger's syndrome (with IQ < 70)	11 (0)	-	
Number with PDD-NOS (with IQ < 70)	4 (1)	-	

ASD: autism spectrum disorder, M: male, F: female, IQ: intelligence quotient; PDD-NOS: pervasive developmental disorder not otherwise specified. Data are means ± SD unless otherwise specified. Differences in clinical characteristics were analyzed using the χ^2 test for gender and the Mann-Whitney U test for age.

57 questions in eight domains of the characteristics of children with PDD, which was developed by the Autism Society Japan. The clinicians who diagnosed the individuals were trained in the use of PARS. Twenty individuals met the full criteria for autistic disorder, 11 met the criteria for Asperger syndrome and four for PDD-not otherwise specified (PDD-NOS). Among the patients with ASD, 11 had a low intelligence quotient (IQ) (< 70). The schizophrenia cohort consisted of 45 patients with schizophrenia and 45 age- and sex-matched healthy controls (Table 2). Each patient with schizophrenia received a consensus diagnosis by at least two trained psychiatrists according to the DSM-IV-TR criteria using the structured clinical interview (SCID) for DSM-IV.

A detailed description of healthy controls was given in previous reports [35,36]. Briefly, controls were biologically unrelated Japanese participants. Healthy controls were screened using the SCID for the *Diagnostic and Statistical Manual, Fourth Edition*, Axis I Disorders, Non-Patient version (SCID-I/NP) and were excluded if they (1) had neurological or medical conditions that could potentially affect the central nervous system, (2) had any psychiatric diseases and/or received psychiatric medication, (3) had first- or second-degree relatives with psychiatric disease or (4) presented with an IQ < 70. IQ data were collected using the Japanese version of the full-scale Wechsler Adult Intelligence Scale (WAIS)-III or the full-scale Wechsler Intelligence Scale for Children-Third Edition (WISC-III) [37,38].

Following description of the study, written informed consent was obtained from each individual (or, when appropriate, his/her guardians). This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and was approved by the ethics committee at Osaka University.

Immortalization of lymphocytes and RNA extraction
Isolation of lymphocytes from blood and lymphocyte immortalization using Epstein-Barr virus (EBV) were entrusted to SRL of Tokyo, Japan. Immortalized, patient-derived lymphocytes were grown in culture media supplemented with 20% fetal bovine serum. Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). The total RNA yield was determined by absorbance at 260 nm, and RNA quality was analyzed using agarose gel electrophoresis.

DNase treatment and reverse transcriptase reaction
Total RNA was treated with DNase to remove contaminating genomic DNA using DNase Treatment & Removal Reagents (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Total RNA (10 μg) treated with DNase was used in a 50-μL reverse transcriptase reaction to synthesize cDNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, total RNA (10 μg) was denatured with 1 mM deoxyribonucleotide triphosphate (dNTP) and 5 ng/μL random hexamers at 65°C for 5 minutes. After the addition of 10xRT buffer (20 mM Tris-HCl (pH 8.4) and 50 mM KCl final concentration; Invitrogen), $MgCl_2$ (5 mM final concentration), dithiothreitol (10 mM final concentration), RNaseOUT Recombinant Ribonuclease Inhibitor (100 U; Invitrogen) and SuperScript III Reverse Transcriptase (125 U; Invitrogen), the reaction mixture was incubated at 25°C for 10 minutes, at 42°C for 40 minutes and at 70°C for 15 minutes. RNase H (5 U) was added to the reaction mixture and incubated at 37°C for 20 minutes to stop the reaction.

Real-time quantitative RT-PCR
The Pre-Developed TaqMan Assay Reagent kit (Applied Biosystems, Foster City, CA, USA) was used to measure mRNA expression levels of *NLGN3, NLGN4, NRXN1, SHANK3, MeCP2, NHE9, AKT1* and housekeeping genes (*β-actin* and *TBP*). Primers were purchased from Applied Biosystems (gene name: assay ID, transcript ID, target region; *NLGN3*: Hs01043809_m1, NM_181303.1, Exon4-5; *NLGN4*: Hs00535592_m1, NM_020742.2, Exon1-2; *NRXN1*: Hs00985123_m1, NM_001135659.1, Exon22-23; *SHANK3*: Hs01586468_m1, NM_001080420.1, Exon22-23; *MECP2*: Hs00172845_m1, NM_004992.3, Exon2-3; *NHE9*: Hs00543518_m1, NM_173653.3, Exon7-8; *AKT1*: Hs00920503_m1, NM_001014432.1, Exon13-14; β-actin: 4326315E, NM_001101, no region indicated; *TBP*: 4326322E, NM_003194, no region indicated). Expression levels of these genes were measured by real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with a 384-well format as previously described [39,40]. Each 20-μL PCR reaction contained 6 μL of cDNA, 900 nM concentrations of each primer, a 250 nM concentration of probe and 10 μL of

Table 2 Demographic information for schizophrenia and control cohorts[a]

Demographics	Schizophrenia (n = 45)	Controls (n = 45)	P value
Sex, M/F	26/19	26/19	$\chi^2 = 0$ (1, N = 90), P = 1.0
Mean age, years (± SD)	37.9 (1.6)	38.1 (1.7)	U = 988.5, P = 0.9, Z = -0.2
Age range, years	21 to 65	21 to 65	
Estimated premorbid IQ (JART50)	100.8 (9.3)	105.4 (8.4)	U = 687, P = 0.009, Z = -2.6

M: male, F: female, IQ: intelligence quotient; JART50: Japanese Adult Reading Test: Japanese version of the National Adult Reading Test. Data are means ± SD unless otherwise specified. Differences in clinical characteristics were analyzed using the χ^2 test for gender and the Mann-Whitney U test for age.

TaqMan Universal PCR Master Mix containing Ampli-Taq Gold DNA Polymerase and AmpErase Uracil N-gly-cosylase (all from Applied Biosystems), as well as dNTP with deoxyuridine triphosphate, passive reference and optimized buffer components. The PCR cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 59°C or 60°C for 1 minute. PCR data were obtained by using Sequence Detector software (SDS version 2.1; Applied Biosystems) and quantified using a standard curve method. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at threshold (C_t) (where the fluorescence generated within a reaction crossed the threshold) versus the quantity of RNA. All samples were measured using a single plate per target gene, and their C_t values were in the linear range of the standard curve. Sample quantities were predicted by C_t values. Experiments were typically performed three times in triplicate, and each gene expression level was taken as the average of three independent experiments. The individual expression level of each target gene normalized by a housekeeping gene (raw target gene expression level divided by raw housekeeping gene expression level) was used for statistical analysis.

Statistical analyses

Statistical analyses were carried out using SPSS for Windows version 16.0 software (SPSS Japan Inc., Tokyo, Japan). Group comparisons of demographic data were performed using the c^2 test for one categorical variable (sex) or the Mann-Whitney U test for continuous variables as appropriate. Differences in mRNA transcript levels between the groups were analyzed using the Mann-Whitney U test. The Bonferroni correction for multiple tests was applied to assess the mRNA transcript levels on the number of genes (five). All P values reported are based on two-tailed tests. Statistical significance was defined as $P < 0.05$.

Results

Standard curves for the seven target genes (*NLGN3, NLGN4, NRXN1, SHANK3, MeCP2, NHE9* and *AKT1*) and the two housekeeping genes (*β-actin* and *TBP*) were prepared using serial dilutions (1:4) of pooled cDNA from 300 ng of total RNA derived from immortalized lymphoblasts (Figure 1). The R^2 values of the standard curves were more than 0.99 (*NLGN3, MeCP2, NHE9, AKT1, β-actin* and *TBP*), 0.87 (*SHANK3*), 0.64 (*NRXN1*) and 0.63 (*NLGN4*). Although the *SHANK3* gene expression was relatively low, it was measurable in our sample. On the other hand, we did not further analyze *NLGN4* and *NRXN1* gene expression, as the expression levels of
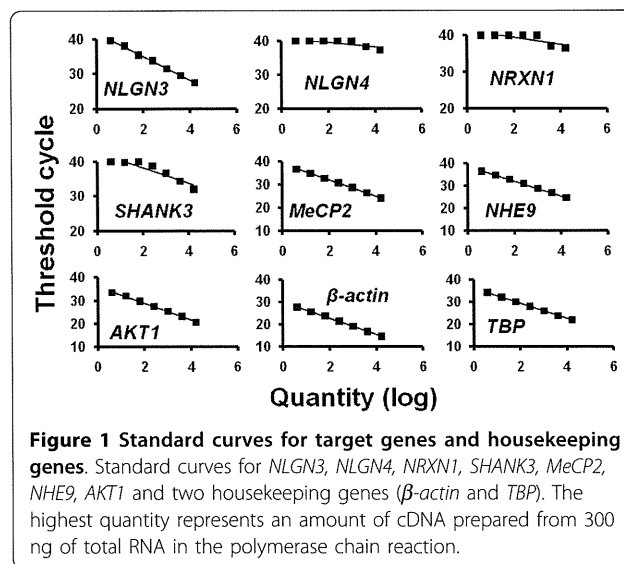
Figure 1 Standard curves for target genes and housekeeping genes. Standard curves for *NLGN3, NLGN4, NRXN1, SHANK3, MeCP2, NHE9, AKT1* and two housekeeping genes (*β-actin* and *TBP*). The highest quantity represents an amount of cDNA prepared from 300 ng of total RNA in the polymerase chain reaction.

the two genes were too low to quantify using this method.

Using immortalized lymphoblastoid cells from 35 individuals with ASD and 35 controls, we quantified the mRNA expression levels of the *NLGN3, SHANK3, NHE9, MeCP2* and *AKT1* genes normalized by two housekeeping genes, *β-actin* and *TBP* (Figure 2). The mRNA expression levels of the *NLGN3* gene normalized by *β-actin* or *TBP* were decreased by 35% or 26%, respectively, in individuals with ASD (*β-actin*: $P =$
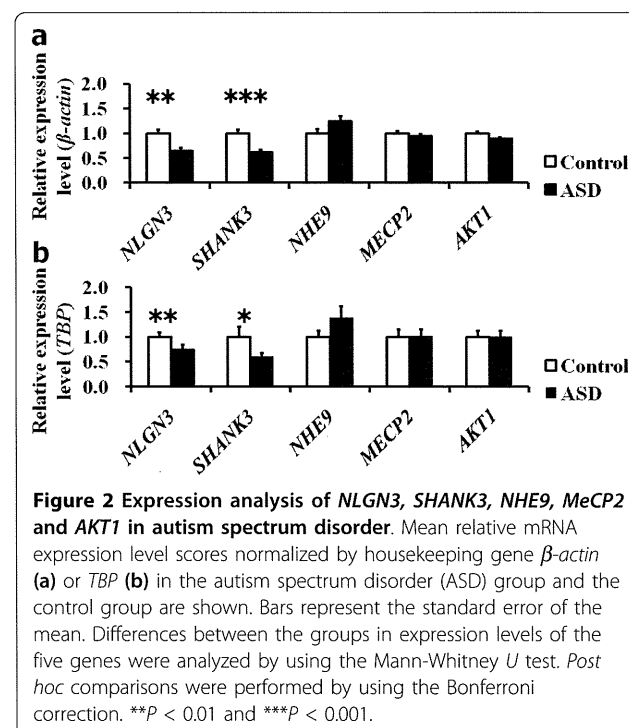
Figure 2 Expression analysis of *NLGN3, SHANK3, NHE9, MeCP2* and *AKT1* in autism spectrum disorder. Mean relative mRNA expression level scores normalized by housekeeping gene *β-actin* (a) or *TBP* (b) in the autism spectrum disorder (ASD) group and the control group are shown. Bars represent the standard error of the mean. Differences between the groups in expression levels of the five genes were analyzed by using the Mann-Whitney U test. Post hoc comparisons were performed by using the Bonferroni correction. **$P < 0.01$ and ***$P < 0.001$.

0.00024; *TBP*: *P* = 0.00089). The mRNA expression levels of the *SHANK3* gene normalized by *β-actin* or *TBP* were also decreased in individuals with ASD by 39% or 40%, respectively (*β-actin*: *P* = 0.000036; *TBP*: *P* = 0.0061). The mRNA expression levels of the *NHE9* gene were increased by 24% (*P* = 0.052: normalized by *β-actin*) and 39% (*P* = 0.048: normalized by *TBP*). There was no significant difference in mRNA expression levels of the *MeCP2* gene normalized by *β-actin* or *TBP* between the two groups (*P* > 0.1). The mRNA expression levels of the *AKT1* gene were decreased by 11% (*P* = 0.03: normalized by *β-actin*); however, those levels were not altered when normalized by *TBP* (*P* = 0.45). After correction for multiple tests, mRNA expression levels of *NLGN3* and *SHANK3* remained significantly lower in individuals with ASD than in healthy controls (*NLGN3*: corrected *P* = 0.0012, normalized by *β-actin*, corrected *P* = 0.0045, normalized by *TBP*; *SHANK3*: corrected *P* = 0.00018, normalized by *β-actin*, corrected *P* = 0.03, normalized by *TBP*). However, the altered expression level of *NHE9* or *AKT1* was no longer significant after the correction for multiple tests (*P* > 0.1).

We next measured *NLGN3* and *SHANK3* mRNA expression levels in immortalized lymphoblastoid cells from 45 patients with schizophrenia and 45 healthy controls to examine the disease specificity of the differential expression levels between patients and healthy controls (Figure 3). We found that the mRNA expression levels for these two genes normalized by *β-actin* or *TBP* were not significantly different between patients with schizophrenia and healthy controls (*P* > 0.2). These results suggest that reduced levels of *NLGN3* and *SHANK3*

Figure 3 Expression analysis of the *NLGN3* and *SHANK3* genes in patients with schizophrenia. Mean relative mRNA expression level scores normalized by housekeeping gene *β-actin* **(a)** or *TBP* **(b)** in the schizophrenia group and the control group are shown. Bars represent standard error of the mean. Differences in expression levels of the two genes between the groups were analyzed by using the Mann-Whitney *U* test.

mRNA expression might be associated with ASD but not with schizophrenia.

Discussion

In this study, we found that the mRNA expression levels of *NLGN3* and *SHANK3* were significantly lower in individuals with ASD than in healthy controls. Mutations of causal genes are rare, and they have been found to be associated with specific types of ASD. Our findings suggest that not only rare mutations of the causal genes but also functional alterations in the transcriptional activity of these genes might be associated with the pathophysiology of ASD. The *NLGN3* and *SHANK3* genes are synapse-related genes and were found to be affected in ASD, whereas other genes, including *NHE9* and *MeCP2*, do not play major roles at the synapse and were not found to be affected in ASD. These findings suggest that impairments in synaptic function might be associated with the pathophysiology of ASD.

Reduced expression of the *NLGN3* and *SHANK3* genes in lymphoblasts of individuals with ASD is consistent with previous reports indicating that mutations of these genes cause reduced expression or loss of function of the protein. Since the *NLGN3* gene is located in chromosome X, there may be expressional difference between genders. However, no significant difference of *NLGN3* gene expression normalized by *β-actin* or *TBP* was observed with regard to gender in healthy controls or individuals with ASD (*P* > 0.05). This might be due to inactivation of one X chromosome in females [41]. There are several possibilities that might explain the reduced expression of the *NLGN3* and *SHANK3* genes in ASD. First, our sporadic ASD cases might have mutations, polymorphisms or copy number variations in the *NLGN3* or *SHANK3* genes, which could result in reduced expression of these genes. Second, mutations or polymorphisms in genes that regulate the expression of *NLGN3* or *SHANK3* might contribute to the observed reduction in expression of the *NLGN3* or *SHANK3* genes. To our knowledge, although the regulation of *NLGN3* by other genes has not been reported, there are some reports in the literature describing the regulation of *SHANK3* gene expression. For example, *SHANK3* expression is regulated by DNA methylation [42,43]. In addition, *SHANK3* is one of the predicted targets of dysregulated microRNA (miRNA), and altered miRNA expression levels were found in postmortem brain from autism patients [44]. Further epigenetic analyses might elucidate the mechanisms of reduced *SHANK3* expression.

Some findings of gene expression in lymphoblastoid cell lines are in conflict with those of previous studies. For example, Beri *et al.* [42] reported that *SHANK3* is not expressed in EBV-transformed human lymphoblastoid cell lines in an investigation of tissue-specific
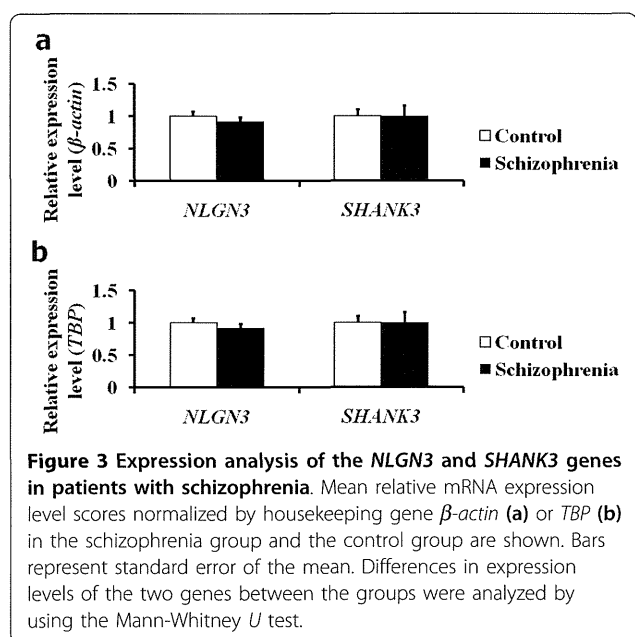
SHANK3 gene expression and DNA methylation. By using lymphoblastoid cells from autism patients, Talebizadeh *et al.* [8] detected novel splice isoforms of *NLGN4*. There are methodological differences between previous studies and our study. *SHANK3* gene expression in the previous study [42] was analyzed by using a conventional RT-PCR method; however, we measured the expression levels of *SHANK3* gene by using a real-time qRT-PCR method (the TaqMan method). Furthermore, the expression level of *SHANK3* was relatively low, which is shown in the standard curve in Figure 1. It is possible that the sensitivity of our real-time qRT-PCR method to detect the *SHANK3* gene expression level might be higher than that of a conventional RT-PCR method. On the other hand, we could not quantitatively measure the *NLGN4* and *NRXN1* genes by using the real-time qRT-PCR method. However, there were slight expressions of these genes in lymphoblastoid cell lines when we used a large quantity of cDNA for the real-time qRT-PCR (Figure 1). Unfortunately, the small expression levels of these genes made it impossible to quantitatively measure the gene expressions in our sample. This may explain possible discrepancies of the gene expression findings of previous studies and our results.

There are several limitations of this study. First, our positive results might have arisen from sample bias due to non-age-matched samples, although the Japanese are a relatively homogeneous population, so the use of non-age-matched samples is unlikely to explain our findings. Second, our sample size might not be small for type I errors but small for type II errors. There is a possibility of type II errors in mRNA expression differences of *NHE9*, *MECP2* and *AKT1* between individuals with ASD and healthy controls and expression differences of *NLGN3* and *SHANK3* between individuals with schizophrenia and healthy controls. In particular, *NHE9* might be increased in individuals with ASD, as the expression level of *NHE9* was marginally significant before correction for multiple testing. Thus, replication studies using a larger sample size are needed before a firm conclusion can be drawn. Third, we did not perform a mutation search for the examined genes in our sample to replicate the association between the examined genes and ASD and how the causal or risk variants of the genes regulate the gene expression. As the previous evidence for candidate genes of ASD are based on rare mutations and/or copy number variations of the genes, it might be difficult to find a mutation in our 35 individuals with ASD for analysis of the variant effects on the gene expression in this study. A mutation search study of these candidate genes should be done in future studies. Fourth, the IQ scores in the ASD group were lower than those in the healthy control group, so reduced gene expression could be related to lower IQ. However, lower expression

of the *NLGN3* or *SHANK3* genes was not found in individuals with schizophrenia who had lower premorbid IQ scores, and no expression difference was observed in individuals with ASD and mental retardation versus individuals with ASD but without mental retardation (data not shown). Taken together, the reduced gene expression in ASD might be specific to ASD, although other neuropsychiatric diseases, such as attention-deficit/hyperactivity disorder, mental retardation, major depression and bipolar disorder, should be examined in future studies. The ASD cases in this study were consistent with idiopathic autism diagnosed on the basis of clinical features. We did not include individuals with Rett syndrome and the other syndromic autisms, such as multiple sclerosis, which could explain why we did not find altered expression of *MeCP2* in this cohort. Our results suggest that the *MeCP2* gene may not be associated with the common pathology of ASD, while *NLGN3* and *SHANK3* may be. Because lymphoblastoid cell lines are not neuronal cells, some of our findings might not reflect the pathophysiology in ASD brains. Further studies investigating these limitations are warranted.

Conclusions

Our study reveals reduced levels of *NLGN3* and *SHANK3* mRNA expression in lymphoblastoid cell lines derived from individuals with ASD, but not from those of individuals with schizophrenia. These results are consistent with findings that rare mutations of these genes in specific cases cause loss of function, suggesting that reduction of *NLGN3* and *SHANK3* mRNA expression could be related to the pathophysiology of ASD in a substantial population of patients. Although there are several limitations present in this study, lymphoblastoid cell lines may still allow investigation of the pathophysiology of ASD. Further analyses are required, such as a mutation analysis of the *NLGN3* and *SHANK3* genes and the genes regulating their expression, in addition to studies designed to elucidate the mechanisms of this reduced expression.

Author details

[1]Department of Psychiatry, Osaka University Graduate School of Medicine, D3, 2-2, Yamadaoka, Suita, 565-0871, Osaka, Japan. [2]CREST (Core Research for Evolutionary Science and Technology) of JST (Japan Science and Technology Agency), 4-1-8, Honcho, Kawaguchi, Saitama, 332-0112, Japan. [3]Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, D3, 2-2, Yamadaoka, Suita, Osaka, 565-0871, Japan. [4]Department of Molecular Neuropsychiatry, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka, 565-0871, Japan. [5]Division of Developmental Neuroscience, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, 2-2, Yamadaoka, Suita, Osaka, 565-0871, Japan.

Authors' contributions

RH supervised the entire project; collected the data; wrote the manuscript; was critically involved in the design, analysis and interpretation of the data; and was responsible for performing the literature review. YY was critically involved in the collection and analysis of the data, contributed to the editing of the final manuscript and contributed intellectually to the interpretation of the data. HY, SU and AI were involved in the mRNA measurements and collection of the majority of the data. KO, MF, IM, MTan and MTak were heavily involved in the collection of the majority of the data and contributed intellectually to the interpretation of the data. All authors reviewed the manuscript before submission and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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