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Archival Report

Biological Psychiatry

Utility of Scalp Hair Follicles as a Novel Source of Biomarker Genes for Psychiatric Illnesses

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ABSTRACT

BACKGROUND: Identifying beneficial surrogate genetic markers in psychiatric disorders is crucial but challenging.

METHODS: Given that scalp hair follicles are easily accessible and, like the brain, are derived from the ectoderm, expressions of messenger RNA (mRNA) and microRNA in the organ were examined between schizophrenia (*n* for first/second = 52/42) and control subjects (*n* = 62/55) in two sets of cohort. Genes of significance were also analyzed using postmortem brains (*n* for case/control = 35/35 in Brodmann area 46, 20/20 in cornu ammonis 1) and induced pluripotent stem cells (*n* = 4/4) and pluripotent stem cell-derived neurospheres (*n* = 12/12) to see their role in the central nervous system. Expression levels of mRNA for autism (*n* for case/control = 18/24) were also examined using scalp hair follicles.

RESULTS: Among mRNA examined, *FABP4* was downregulated in schizophrenia subjects by two independent sample sets. Receiver operating characteristic curve analysis determined that the sensitivity and specificity were 71.8% and 66.7%, respectively. *FABP4* was expressed from the stage of neurosphere. Additionally, microarray-based microRNA analysis showed a trend of increased expression of *hsa-miR-4449* ($p = .0634$) in hair follicles from schizophrenia. *hsa-miR-4449* expression was increased in Brodmann area 46 from schizophrenia ($p = .0007$). Finally, we tested the expression of nine putative autism candidate genes in hair follicles and found decreased *CNTNAP2* expression in the autism cohort.

CONCLUSIONS: Scalp hair follicles could be a beneficial genetic biomarker resource for brain diseases, and further studies of *FABP4* are merited in schizophrenia pathogenesis.

Keywords: Autism, *CNTNAP2*, FABP4, *hsa-miR-4449*, MicroRNA, Schizophrenia

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The disease mechanisms underlying psychiatric illnesses remain largely undetermined. Great efforts have gone into identifying novel biomarkers that would assist in the development of objective diagnostic tools and novel therapeutic and prophylactic interventions, as well as facilitate the subdivision of disease states, based on pathogenesis, for optimal drug selection. There are, however, major obstacles in the search for novel biomarkers, primarily the difficulty in obtaining brain tissue from living donors and the lack of accurate experimental animal models. Brain is an ectodermal tissue and shares its developmental origins with scalp hair follicles, which are readily accessible miniorgans within the skin. Despite their shared embryonic origins, hair follicles have not previously been utilized as a bio-resource in the hunt for proxy genes in psychiatric diseases. In the current study, we first examined whether schizophrenia-relevant genes, namely those related to the γ-aminobutyric acid (GABA)ergic system (1–3), myelin (3–5), and fatty acids (6–11), are expressed in hair follicles and if expressed whether expression is differential between cases and control subjects, using an exploratory sample set. Next, we attempted to validate any differential expression and examine the effects of potential confounding factors using a second independent sample set. We then analyzed the identified biomarker candidate *FABP4*/fatty acid binding protein 4 (FABP4) expression in serum, postmortem brain samples, induced pluripotent stem cells (iPSCs), and iPSC-derived neurospheres. In addition to messenger RNA (mRNA), we also examined the expression levels of microRNA (miRNA) in hair follicles, postmortem brains, iPSCs, and iPSC-derived neurosphere samples from patients with schizophrenia and control subjects. Lastly, we tested candidate gene expression in hair follicles from patients with autism. Based on the results of our comprehensive analysis, we proposed scalp hair follicles as a beneficial genetic resource for schizophrenia and autism in the search for potential biomarkers.

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METHODS AND MATERIALS

Scalp Hair Follicle Samples

All samples were collected from ethnic Japanese within Japan. The first set of exploratory scalp hair follicle samples for schizophrenia and control subjects was derived from residents in the northern district of Kanto, while the confirmatory second set came from the Tokyo area. Diagnoses were made by at least two experienced psychiatrists, using DSM-IV criteria. Demographic data for scalp hair follicle samples derived from schizophrenia are described in Table 1. The scalp hair follicle samples from autism participants and control subjects were collected from the Chubu area. The diagnosis of autism spectrum disorder was made using the DSM-IV-TR criteria. We then administered the Autism Diagnostic Interview-Revised (ADI-R) (12) to 14 of 18 cases and made a confirmed diagnosis of autism for those 14 cases. Interviews for the ADI-R were conducted by experienced child psychiatrists who are licensed to use the Japanese version of the ADI-R (13). Demographic data relating to scalp hair follicle samples for autism are described in Table 1.

RNA Extraction and Quantification

Ten hairs were plucked from the scalp of each subject using forceps. The hairs were checked for the presence of a sheath. Hairs were trimmed to approximately 1.5 cm in length, containing the bulb region, and dropped into a 1.5 mL microfuge tube (BM Equipment, Tokyo, Japan) containing RNAlater solution (Ambion, Grand Island, New York). Total RNA was extracted using the RNAqueous-Micro kit (Ambion). Single-stranded complementary DNA (cDNA) was synthesized using SuperScript VILO Master Mix (Invitrogen, Grand Island, New York). Quantitative reverse-transcription PCR (qRT-PCR) analysis of mRNAs was conducted using an ABI7900HT Fast Real-Time PCR System (Applied Biosystems, Grand Island, New York). TaqMan probes were TaqMan Gene Expression Assays products (Applied Biosystems). All qRT-PCR data were captured using the SDS v2.4 (Applied Biosystems). The ratios of relative concentrations of target molecules to the *GAPDH* gene (target molecule/*GAPDH* gene) were calculated. All reactions were performed in triplicate based on the standard curve method.

Statistical Analysis

We used the interquartile range to find outliers. The differences between the 25th (quartile 1) and 75th percentiles (quartile 3) were used to identify extreme values (outliers) in the tails of the distribution. Statistical evaluation was performed by Mann-Whitney *U* test for means between patient and control groups and by Spearman's R test for correlation using SPSS software version 19 (IBM, Tokyo, Japan).

Analyses of miRNA Expressions and Potential Targets of miRNAs

For microarray-based miRNA analysis, we used the miRBase Rel. 18.0 platform (Agilent Technologies, Santa Clara California), capable of measuring 1919 human mature miRNAs in the age-/sex-matched subset of the first hair follicle sample set (Table S1 in Supplement 1). The miRNAs were labeled using the miRNA Complete Labeling Reagent and Hyb Kit (Agilent Technologies) and hybridized to the arrays. Images were scanned with a High-Resolution C scanner (Agilent Technologies) and analyzed using GeneSpring GX (Agilent Technologies). Comparisons of miRNA expression values between schizophrenia and control groups were performed using GeneSpring 12.6 (Agilent Technologies). To normalize the intermicroarray range of expression intensities, the percentile shift method (90th percentile) was used. The genes whose expression data were available in more than 50% of hybridizations were statistically evaluated between schizophrenia and control groups using the two-tailed Mann-Whitney *U* test. For quantification of individual miRNAs, we performed TaqMan-based miRNA qRT-PCR (Applied Biosystems, Grand Island, New York) according to the manufacturer's instructions, using *U6 snRNA* as a control probe. All reactions for miRNA quantification were also performed in triplicate, based on the standard curve method. Statistical evaluation methods were the same as those for mRNA.

Table 1. Demographic Characteristics of Hair Follicle Sample Sets

	Control Subjects	Patients	*p* Value
First Sample Set for Schizophrenia			
n	62	52	
Sex (female/male)	41 / 21	25 / 27	.0518[a]
Age (mean ± SD)	41.26 ± 12.26	50.98 ± 10.86	<.0001[b]
Second Sample Set for Schizophrenia			
n	55	42	
Sex (female/male)	26 / 29	20 / 22	.973[a]
Age (mean ± SD)	46.87 ± 13.56	49.93 ± 12.97	.2777[b]
Duration of illness (mean ± SD)		22.79 ± 14.66	
Autism Sample Set			
n	24	18	
Sex (female/male)	24 / 0	16 / 2	.1777[a]
Age (mean ± SD)	32.60 ± 3.91	25.61 ± 4.95	<.0001[b]

[a]Evaluated by chi-square test.
[b]Evaluated by two-tailed *t* test.

To identify the potential targets of a specific miRNA, we performed in silico analysis using web-based miRNA target prediction methods, TargetScan (http://www.targetscan.org, Release 6.2; Whitehead Institute for Biomedical Research, Cambridge, Massachusetts) and miRDB (http://mirdb.org/miRDB/; Washington University School of Medicine, St. Louis, Missouri).

Immunohistochemistry

The plucked hairs were rinsed briefly in phosphate-buffered saline and dropped into a 1.5 mL microfuge tube containing 1 mL of 10% neutral-buffered formalin (4°C, 1 hour). The fixed hairs were pre-embedded in 4% agarose (Sigma-Aldrich, St Louis, Missouri) in phosphate-buffered saline, pH 7.4. At this point, it was possible to orientate the hairs into their desired position for either longitudinal or transverse sectioning. Blocks were embedded in capsules, which were filled with O.C.T. compound (Sakura Finetek, Tokyo, Japan). Cryostat sections (8 μm thick) of plucked hair follicles were processed for immunohistochemistry. The sections were blocked with 10% goat serum in .05 mol/L Tris buffered saline plus .05% Tween 20 (TBST), followed by three rinses in TBST (20 min each). The primary antibodies were applied for overnight at 4°C. After three washes in TBST (20 min each), secondary antibodies were applied to sections at room temperature (1 hour). Slides were counterstained with 4',6-diamidino-2-phenylindole to highlight nuclei. After washing in TBST, the slides were mounted in PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific, Waltham, Massachusetts). Fluorescent signals were detected using a confocal laser-scanning microscope FV1000 (Olympus, Tokyo, Japan).

Antibodies

See Supplementary Methods and Materials in Supplement 1.

Analysis of FABP4 Protein Levels in Serum

See Supplementary Methods and Materials in Supplement 1.

Postmortem Brain Analysis

See Supplementary Methods and Materials in Supplement 1.

Establishment of iPSC Lines

Dermal fibroblasts (human dermal fibroblasts) from the facial dermis of a 36-year-old Caucasian female subject (Cell Applications, Inc., San Diego, California) were used to establish control iPSCs 201B7 and YA9 (14). The remaining control iPSCs, WD39 and KA23, were generated from a 16-year-old Japanese female subject (15) and a 40-year-old Japanese male subject (Matsumoto, Ph.D., *et al.*, personal communication, 2013), respectively. The 201B7 iPSCs were kindly provided by Yamanaka, M.D., Ph.D., Kyoto University (14). The iPSCs YA9, WD39, and KA23 have been described in a previous report (15). The schizophrenia derived iPSCs from patients with 22q11.2 deletions SA001 and KO001 were generated from Japanese female subjects aged 37 and 30 years old, respectively (see Clinical History in Supplement 1).

The maintenance of human dermal fibroblasts, lentiviral production, retroviral production, infection, stem cell culture, and characterization were performed as described previously (15).

In Vitro Neural Differentiation of Induced Pluripotent Stem Cells

The iPSCs were plated in T75 flasks after dissociation into single cells and cultured for 14 days in neural culture medium supplemented with leukemia inhibitory factor (Merck Millipore, Darmstadt, Germany) and basic fibroblast growth factor (Peprotech, Rocky Hill, New Jersey). Neurospheres were passaged repeatedly by culturing in the same manner (16,17).

Comparative Genomic Hybridization Array Analysis

See Supplementary Methods and Materials in Supplement 1.

Ethical Issues

This study was approved by the Ethics Committees of RIKEN and all participating institutes, including the Keio University School of Medicine, an ethical committee for skin biopsy and iPSC production (approval No. 20080016), and conducted according to the principles expressed in the Declaration of Helsinki. All control subjects and patients gave informed, written consent to participate in the study after being provided with and receiving an explanation of study protocols and objectives.

RESULTS

Expression of mRNA in Scalp Hair Follicles from Schizophrenia and Control Subjects

Gene expression profiles of schizophrenia postmortem brains have been well studied. However, studies have been hampered by uncontrollable confounding factors associated with postmortem brains and an inaccessibility of brain tissue from living donors. Therefore, we set out to analyze gene expression in hair follicles. Previous studies provide substantial support for reduced expression of genes related to oligodendrocyte and GABAergic systems in schizophrenia pathology (1–4). In addition, our (6,7,9) and other studies (8,10,11) on *FABPs* (genes for fatty acid binding proteins) raise the possibility of disturbed lipid metabolism in the susceptibility to this disease. Based on these findings, we selected 22 genes: 8 from the GABAergic system, 9 with myelin relevance, and 5 with lipid relevance (Table 2). The amount of mRNA from an individual subject's hair follicles was not enough for a systemic cDNA microarray. We used *GAPDH* as an internal control. An exploratory scalp hair follicle sample panel (the first sample set) consisted of samples from 52 patients with schizophrenia and 62 control subjects (Table 1). qRT-PCR analysis showed that seven genes, namely *CALB2*, *SST*, *CNP*, *PMP22*, *FABP4*, *FABP7*, and *FAAH* were differentially expressed ($p < .05$) in samples from schizophrenia compared with control subjects (Table 2; Figure S1 in Supplement 1).

To replicate the finding, we examined the expression levels of these seven genes using an age-/sex-matched, independent confirmatory set (a second sample set) composed of 42 patients with schizophrenia and 55 control subjects (Table 1). Of the seven genes, only *FABP4* showed significantly decreased expression (an average reduction of 43% compared with a reduction of 40% in the first set of samples) in schizophrenia samples (Figure 1A; Table 2). Correlation analyses demonstrated no significant effects for age, dose of

Table 2. List of Examined Genes and Their Expression in the First and Second Scalp Hair Follicle Sample Sets from Schizophrenia

			First Sample Set			Second Sample Set		
			Mean ± SD of Corresponding Gene / *GAPDH*			Mean ± SD of Corresponding Gene / *GAPDH*		
Gene Category	Gene Symbol	Assay ID[a]	Control (n = 62)	Schizophrenia (n = 49)	p Value[b]	Control (n = 62)	Schizophrenia (n = 49)	p Value[b]
GABAergic System	*GAD1*	Hs01065893_m1	.881 ± .598	1.119 ± .707	.118			
	GAD2	Hs00609534_m1	Not detectable					
	GABRA1	Hs00168058_m1	2.347 ± 2.761	.832 ± .964	.378			
	GABRD	Hs00181309_m1	1.055 ± .758	.945 ± .618	.666			
	SLC6A1	Hs01104475_m1	1.047 ± .830	.985 ± .555	.682			
	PVALB	Hs00161045_m1	1.067 ± .569	1.074 ± .669	.87			
	CALB2	Hs00418693_m1	1.024 ± .355	1.163 ± .303	.037[d]	.715 ± .373	.857 ± .300	.095[c]
	SST	Hs00356144_m1	.626 ± .549	1.052 ± .923	.028[d]	.910 ± .683	1.812 ± 1.802	.151[c]
Myelin Relevance	*APC*	Hs01568269_m1	1.001 ± .243	.939 ± .233	.131			
	CLDN11	Hs00194440_m1	.860 ± .605	.984 ± .854	.862			
	CNP	Hs00263981_m1	1.148 ± .336	.985 ± .186	.002[d]	.928 ± .415	1.052 ± .210	.456
	CSPG4	Hs00361541_g1	.976 ± .536	1.050 ± .364	.252			
	MAG	Hs01114387_m1	Not detectable					
	NES	Hs00707120_s1	1.018 ± .496	1.013 ± .403	.98			
	OLG2	Hs00300164_s1	Not detectable					
	PMP22	Hs00165556_m1	1.006 ± .370	.804 ± .261	.003[d]	.807 ± .410	.844 ± .400	.987
	SOX10	Hs00366918_m1	1.072 ± .748	.984 ± .508	.99			
Lipid Relevance	*FABP3*	Hs00997360_m1	.763 ± .486	.807 ± .372	.292			
	FABP4	Hs01086177_m1	1.050 ± .470	.653 ± .251	<.0001[d]	1.138 ± .708	.650 ± .232	**<.001**
	FABP5	Hs02339439_g1	1.118 ± .215	1.084 ± .179	.312			
	FABP7	Hs00361426_m1	.562 ± .332	1.018 ± .744	.003[d]	.519 ± .372	.530 ± .355	.754
	FAAH	Hs01038660_m1	1.008 ± .344	.857 ± .221	.013[d]	.836 ± .303	.753 ± .281	.180[c]
Control	*GAPDH*	Hs02758991_g1						

GABA, gamma-aminobutyric acid.

[a]Probe ID in TaqMan Gene Expression Assay system.

[b]Evaluated by two-tailed Mann-Whitney U test.

[c]For these analyses, only 49 control and 36 schizophrenia samples were available.

[d]Significant changes.

antipsychotics [haloperidol equivalent (18,19)], or duration of illness on the expression levels of *FABP4* (Figure S2A–C in Supplement 1). Since serum levels of Fabp4 were reported to be affected by nutritional fluctuations in mice (that is, suppressed by feeding) (20), we examined the effect of sampling time after the last meal on *FABP4* expression in hair follicles and found no significant change (Figure S2D in Supplement 1). Nor did we detect an effect for sex on *FABP4* levels: male control versus female control subjects, p = .950; male schizophrenia versus female schizophrenia subjects, p = .360; male (control + schizophrenia subjects) versus female (control + schizophrenia subjects), p = .387; all evaluated by the Mann-Whitney U test.

Circulating FABP4 is known to be associated with metabolic markers (21,22), so we examined the effects of weight, height, body mass index, and body fat percentage on *FABP4* expression in the second hair follicle sample set (Figure S3 in Supplement 1). None of these factors affected the expression ratios of *FABP4/GAPDH* in hair follicles. Despite the fact that olanzapine alters lipid metabolism (23,24), we detected no significant correlation between *FABP4* expression levels in hair follicles and olanzapine dose (mg/day) in the second set of schizophrenia samples (Spearman's rho = −.2289; 95% confidence interval = −.5258 to .1178; p = .180).

From these results, *FABP4* expression levels in hair follicles would appear to be a robust marker for schizophrenia. Receiver operating characteristic curve analysis determined an optimal cutoff level of .769, based on the minimum distance from the curve to upper left corner (= .191) and area under the curve = .713 (95% confidence interval = .609–.817) (Figure S4 in Supplement 1). With this cutoff level for the *FABP4/GAPDH* mRNA ratio, the sensitivity, specificity, and positive and negative predictive values were 71.8%, 66.7%, 60.9%, and 76.6%, respectively.

Immunohistochemical Analysis of FABP4 in Scalp Hair Follicles

Figure 2A shows the structure of a hair follicle (25,26). Moving inward, a plucked scalp hair consists of the following components: the outer root sheath, companion layer, inner root sheath (IRS), the cortex, and medullar. Each of these components has an epidermal origin and each compartment expresses specific genes from the keratin family (26) (Figure 2B). FABP4 is co-expressed with K71 in the IRS cuticle layer and displays partially overlapping expression with K85 in the cuticle, matrix/precortex, and mid/upper cortex (27). However, FABP4 shows scant co-expression with K14 in the outer root sheath layer (Figure 2C, D). These results indicate that FABP4 is expressed in the IRS and part of the hair cortex.

Expression of FABP4 in Serum and Postmortem Brains

We measured FABP4 protein levels in the same cohort as the second hair follicle sample, using an enzyme-linked immunosorbent assay kit, to see whether serum levels of FABP4 could

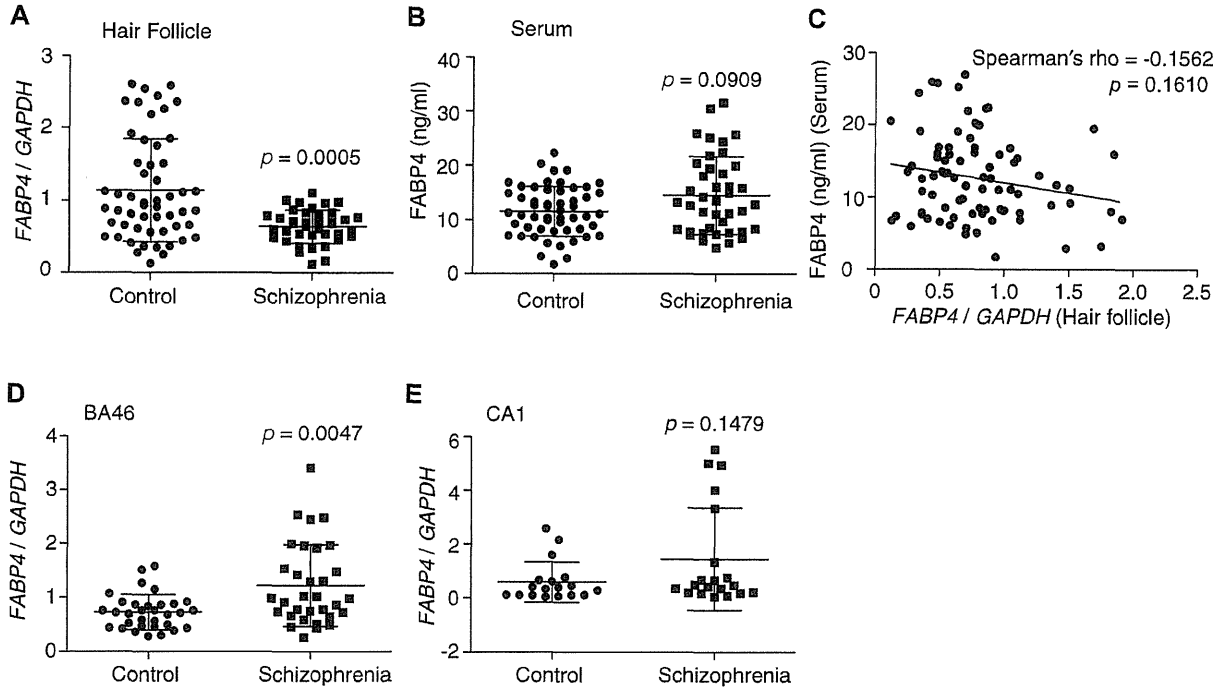


Figure 1. *FABP4*/fatty acid binding protein 4 (FABP4) expression analyses in schizophrenia and control samples. **(A, B, D, E)** Results for hair follicles (the second sample set), serum, and postmortem brain tissue (Brodmann area [BA]46 and cornu ammonis [CA]1) are shown. *GAPDH* was used as an internal control. *p* values were calculated using two-tailed Mann-Whitney *U* test. Horizontal bars show mean ± SD. **(C)** Correlations between relative *FABP4* expression levels in scalp hair follicles and FABP4 levels in serum are also shown. Statistical evaluations were performed using Spearman's rank correlation test.

also be a proxy for schizophrenia. However, the measure did not differ significantly between schizophrenia and control samples, although a trend of increase was seen in schizophrenia (Figure 1B). In addition, using the second sample cohort, there was no significant correlation between serum FABP4 and *FABP4* mRNA levels in hair follicles (Figure 1C). Interestingly, in contrast to findings in mice (20), serum FABP4 levels were not affected by time elapsed after the last meal in either disease or control groups (Figure S5 in Supplement 1).

In postmortem brains, *FABP4* transcript expression was significantly elevated in the frontal cortex (Brodmann area [BA]46) of schizophrenia compared with control samples (p = .0047) (Figure 1D), suggesting its role in schizophrenia pathophysiology. Expression of *FABP4* in hippocampus cornu ammonis 1 remained unchanged between schizophrenia and control samples (Figure 1E), implicating region specificity for the function of *FABP4* in schizophrenia. Both of these brain regions showed particularly high expression levels in four schizophrenia samples derived from patients not recorded to have taken particular therapeutic drugs (Table S3 in Supplement 1), although the possibility of drug effects cannot be excluded.

Expression Analysis of miRNAs in Scalp Hair Follicles and Postmortem Brains

We further performed microarray-based miRNA analysis and measured the expression levels of 1919 human mature miRNAs using the miRBase Release 18.0 platform (Agilent) in an age- and sex-matched subset of the first hair follicle sample set (Table S1 in Supplement 1). We detected three miRNAs, which satisfied our criteria of an absolute fold change (FC) (schizophrenia group/control group) ≥ 2 and $p < .05$ (by Mann-Whitney *U* test, two-tailed). These were *hsa-miR-4449* (FC = 3.45, p = .0032), *hsa-miR-1237* (FC = 2.55, p = .028), and *hsa-miR-4769-3p* (FC = 2.03, p = .028). In the next step, we tested these three miRNAs in the second hair follicle sample set (Table 1), using qRT-PCR, with U6 small nuclear RNA as a control probe. *hsa-miR-4449* showed a top hit with upregulation, although not to significant levels, in schizophrenia (FC = 1.25, p = .063) (Figure 3A).

In postmortem brains (BA46), *hsa-miR-4449* showed increased expression (p = .0007) in schizophrenia samples (Figure 3B), suggesting possible contribution of this gene also to schizophrenia.

Expression Analysis of *FABP4* and *hsa-miR-4449* in iPSCs and iPSC-Derived Neurospheres

Recently, iPSCs have been used for human disease modeling, particularly in neurological disorders (28–30). We have established iPSCs from control subjects (one line each from four subjects) and schizophrenia patients carrying a 22q11.2 microdeletion (two lines each from two patients) (31) (Figure 4). Then, we established three neurosphere lines from each iPSC line. We chose 22q11.2 deletion carriers for analysis (for comparative genomic hybridization array analysis using the iPSCs, see Supplementary Methods and Materials in Supplement 1), since the 22q11.2 deletion is a well-defined

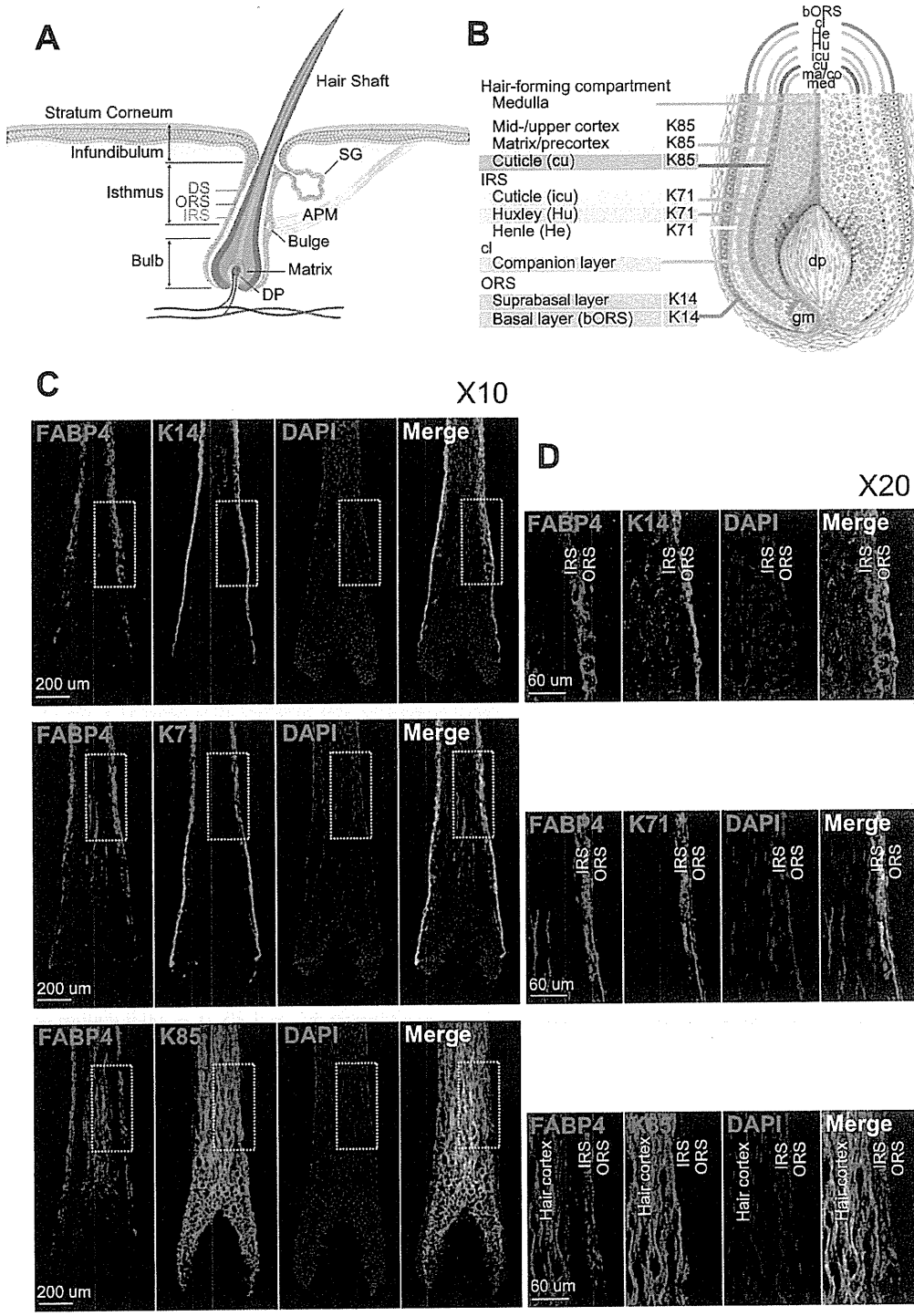


Figure 2. Expression patterns of fatty acid binding protein 4 (FABP4) in scalp hair follicles. **(A)** Schematic illustration showing the structure of hair follicles. **(B)** Schematic presentation of epithelial/hair keratin expression patterns. Keratin K71 is expressed in the three inner root sheath (IRS) layers, while K14 is known as outer root sheath (ORS) keratin. Keratin K85 is present in the hair-forming compartment. **(C)** Immunofluorescent labeling of FABP4 and hair keratins (K14, K71, and K85) in scalp hair follicles. K14 is uniformly expressed throughout the widely stratified follicular ORS. K71 is expressed in all compartments of the hair IRS. Keratin K85 expression extends from the hair matrix to the upper cortex and the hair cuticle. FABP4 is seen in the IRS and part of the hair cortex (merged green and red). 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. **(D)** Magnified picture of **(C)**. APM, arrector pili muscle; cl, companion layer; DP, dermal papilla; DS, dermal sheath; gm, germinative matrix; ma/co, matrix/precortex; med, medulla; SG, sebaceous gland.

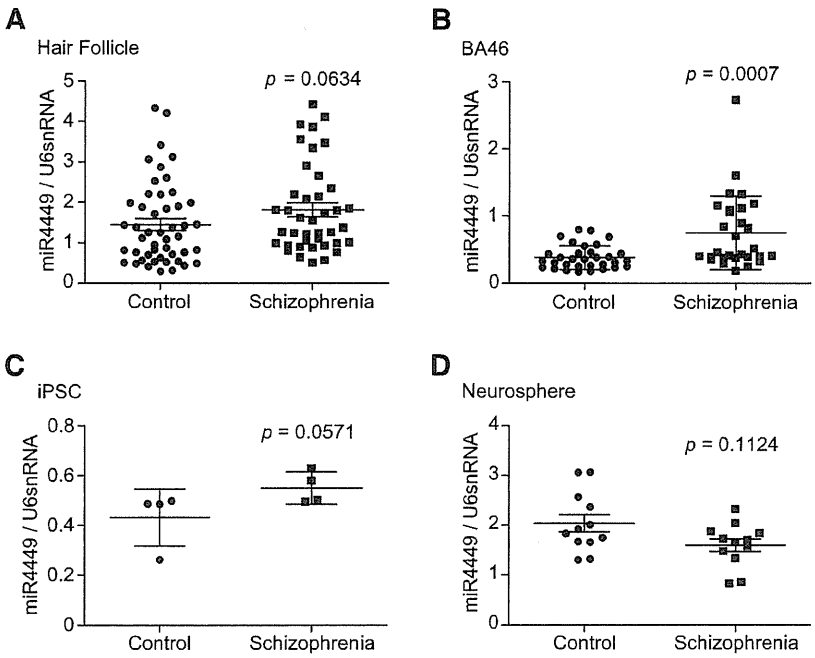


Figure 3. Expression analyses of *hsa-miR-4449* in schizophrenia and control samples. Results from hair follicles (the second sample set) **(A)**, postmortem brains (Brodmann area [BA]46) **(B)**, induced pluripotent stem cells (iPSCs) **(C)**, and neurospheres **(D)** are shown. U6 small nuclear RNA (snRNA) was used as an internal control. *p* values were calculated using two-tailed Mann-Whitney *U* test. Horizontal bars show mean ± SD.

genetic feature with the highest risk for schizophrenia, affecting around .3% of schizophrenia patients (32). The *FABP4* gene shows little expression in iPSCs derived from either control subjects or patients (data not shown). The gene is expressed in neurospheres, suggesting that its expression starts at a very early stage of neuronal development. Neurospheres are composed of free-floating clusters of neural stem or progenitor cells, differentiated from iPSCs. However, *FABP4* expression levels were not significantly different between control subjects and cases (Figure S6 in Supplement 1; expressional variance in the control group was large). Expression of *hsa-miR-4449* showed a trend of upregulation in iPSCs from patients (p = .0571) (Figure 3C); however, there was no differential expression between neurospheres derived from control subjects and cases (Figure 3D).

Examination of Autism Samples

We also performed a preliminary study to examine whether expression patterns of putative autism genes in scalp hair follicles could discriminate between autism and control samples. The sample cohort is shown in Table 1. We selected genes from candidates for autism susceptibility and included *FABP4*, due to the genetic overlap between schizophrenia and autism (33). The remaining genes were *FABP7* (9), *NHE6* (34), *NHE9* (34), *A2BP1* (35), *CADPS2* (36), *AH1* (35), *CNTNAP2* (35), and *SLC25A12* (35). Of the nine genes, only *CADPS2* (p = .0401) and *CNTNAP2* (p = .0212) showed significantly decreased expression in autism-derived samples compared with control follicles (Figure S7 in Supplement 1). It should be noted that the average age of autism subjects was significantly lower than that of control subjects (Table 1) and that *CADPS2* levels showed a positive correlation with age in autism and control + autism groups (Figure S8 in Supplement 1). Therefore, we can only safely nominate *CNTNAP2* level as a potentially valid marker for autism in this study (Figure S9 in Supplement 1). Approximately half of the examined patients were medicated. However, these patients were not outliers in terms of *CNTNAP2* expression in hair follicles; that is, they fell within the mean ± 2SD (detailed data not shown).

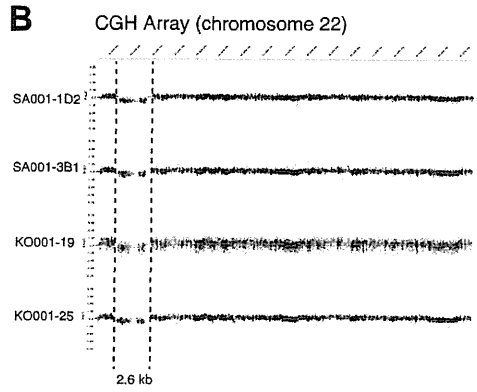
DISCUSSION

We examined and attempted to validate expression levels of schizophrenia and autism candidate genes using scalp hair follicles as a surrogate source of disease markers. Of the protein-coding genes tested that are putative schizophrenia genes, *FABP4* was confirmed to be downregulated in disease samples in our two-stage analyses. Our low rate of replication could be due to two main factors. First, the current sample size is insufficient, which may represent one of the limitations in this study. Another potential reason might be that stable detection of expression levels is dependent on where a particular gene is expressed in the hair follicle. For instance, *FABP4* is expressed in more central portions (IRS and cortex) of the hair follicle and the integrity of these areas may be well maintained during the plucking process, leading to more consistent results.

FABP4, also known as adipocyte-specific fatty acid-binding protein, belongs to the fatty acid-binding protein super family, whose members have molecular masses of approximately 15,000. FABPs are highly conserved cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. It is thought that FABPs are active in fatty acid uptake, transport, and metabolism. In the periphery, FABP4 is highly expressed in adipose tissue and moderately expressed in macrophages, endothelial cells, and bone marrow (37). The protein has been intensively studied in terms of systemic insulin sensitivity and

A

Diagnosis	Sex	Age	Fibroblast	iPSC line No.
Control	F	36	B7	201B7, YA9
Control	F	16	WD	39
Control	M	40	KA	23
Schizophrenia with 22q11del	F	30	KO001	19, 25
Schizophrenia with 22q11del	F	37	SA001	1D2, 3B1



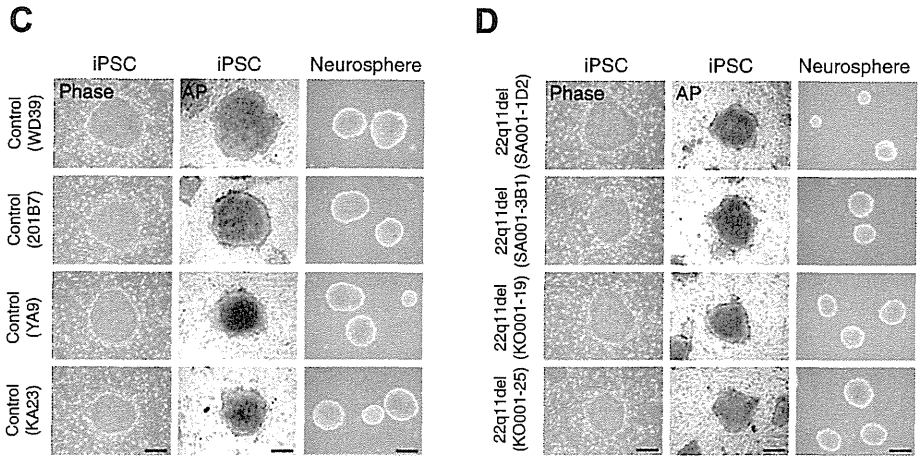


Figure 4. Establishment of iPSCs and iPSC-derived neurospheres from controls and schizophrenia patients with a 22q11.2 deletion (also see ref. 31). **(A)** Demographic data and I.D. information for samples are shown. **(B)** CGH array analysis of chromosome 22 using iPSCs showed that all the iPSC lines derived from the patients carried a 2.6 Mb hemizygous deletion at chromosome 22q11.2. **(C)** Alkaline phosphatase (AP) staining of iPSCs from controls (WD39, 201B7, YA9 and KA23). AP activity was detected using an Alkaline Phosphatase Staining kit (Miltenyi Biotec, Bergisch Gladbach, Germany). **(D)** Those from patients with a 22q11.2 deletion (SA001-1D2, SA001-3B1, KO001-19 and KO001-25). All the iPSC clones were AP-positive showing the pluripotency. Scale bars: phase contrast and AP staining, 400 μm; neurospheres, 150 μm. iPSC, induced pluripotent stem cells.

lipid and glucose metabolism, both of which correlate with inflammatory mechanisms (21). Since the results showing downregulation of *FABP4* in scalp hair follicles from schizophrenia subjects are robust against confounding factors, including those related to metabolic state, our findings are unlikely to represent either metabolic or inflammatory conditions. In addition, our patients had been treated with second-generation antipsychotics, including olanzapine, which often induce metabolic syndrome, but *FABP4* levels in hair follicles were independent of drug dose and duration of illness. Conformingly, there was no significant correlation between serum FABP4 and *FABP4* transcript levels in hair follicles. Therefore, elevated *FABP4* expression in hair follicles may point toward a pathophysiological step in schizophrenia.

In our protocol, all cells in neurospheres expressed the neural markers Nestin, or β3-tubulin, suggesting that our neurospheres consist almost entirely of neural stem or progenitor cells (38). The fact that *FABP4* is expressed in neurospheres may suggest a potential role in neuronal stem cell maintenance or neuronal differentiation or both processes. Although iPSC-derived neurospheres showed no significant differences in *FABP4* expression levels between control and schizophrenia cohorts, before a conclusion can be made, it would be necessary to examine a much larger cohort. According to the Human Protein Atlas database (Knut and Alice Wallenberg Foundation, Stockholm, Sweden; http://www.proteinatlas.org/), *FABP4* transcripts are expressed in neuronal cells (35%) and glial and endothelial cells (65%) of the adult cerebral cortex.

To evaluate whether common genetic variants of *FABP4* determine a predisposition to schizophrenia, we performed a genetic association study using approximately 2000 schizophrenia cases and 2000 age- and sex-matched control subjects with six tag single nucleotide polymorphisms (Supplementary Methods and Materials in Supplement 1). This analysis found no significant allelic or genotypic association (Table S4 in Supplement 1). The *FABP4* gene is composed of two haplotype blocks, based on Gabriel's confidence intervals (39) (Figure S10 in Supplement 1). Haplotype analysis also failed to reveal any significant signals. The exact reasons for the different directional changes seen in hair follicles, serum, postmortem brains, and neurospheres between control and schizophrenia subjects remain unknown. All *FABP* family genes contain a canonical TATA box, followed by a conserved gene structure. The tissue-specific and developmental regulation of *FABP* subtype expression, including that of *FABP4*, is thought to be controlled by unidentified genomic regulatory elements (6,40).

Mechanistically speaking, although not yet confirmed, the *FABP4* may be more central to schizophrenia pathophysiology beyond being a mere biomarker for disease. This is based on the following observations: 1) *FABP4* is expressed in the early neuronal lineage (a current finding); 2) other *FABP* genes are

reported to be associated with schizophrenia (6,7,9,11); and 3) there is evidence linking polyunsaturated fatty acids (endogenous ligands for FABPs) with schizophrenia etiology (41) and brain development (42).

Regarding miRNA, we detected *hsa-miR-4449* from a total of 1919 human mature miRNAs in this study. Although its expression in hair follicles was not significantly altered, expression did show significant upregulation in postmortem brains (BA46) and a trend of increase in iPSCs from schizophrenia samples. Web-based target predictions for *hsa-miR-4449* hit 18 protein-coding genes using TargetScan (Whitehead Institute for Biomedical Research, Release 6.2) (Table S5 in Supplement 1) and 10 protein-coding genes using miRDB (Washington University School of Medicine; http://mirdb.org/miRDB/) (Table S6 in Supplement 1). Between the two programs, the following three genes overlapped: 1) *HIC1*; 2) *RBM4*; and 3) *TOMM40*. Although the predicted roles for these three genes in schizophrenia pathogenesis are not known, *hsa-miR-4449* would make an interesting candidate in future studies, since this miRNA is expressed in early human neurodevelopmental stages such as iPSCs and iPSC-derived neurospheres.

In the analysis of autism-derived scalp hair follicles, we found significant downregulation of *CNTNAP2* in sufferers compared with control subjects and that the results are not affected by age. *CNTNAP2*, which encodes the contactin associated protein-like 2, is one of the strongest autism susceptibility genes with convergent evidence from several independent studies (43).

In the case of schizophrenia, biomarkers are an essential tool, particularly in the early phase of disease onset, such as the prodromal phase or at-risk mental state (44). It would be important to confirm whether *FABP4* expression levels in scalp hair follicles constitute a valid measure for discriminating between those individuals in at-risk mental state who will spontaneously recover and those who will need therapeutic treatment. As a starting point, it is interesting that the decreased *FABP4* levels in schizophrenia-derived hair follicles are not influenced by duration of illness.

In summary, our results provide an original concept for identifying novel disease markers, with potential benefits for the clinical practice of psychiatric medicine, as well as possible applications to other brain disorders. The development of methods that enable the analysis of a transcriptome using hair follicles (~10 samples) would be highly desirable. At the moment, approximately 40 ng of total RNA is extractable from a single hair follicle, but this amount is not enough for currently available cDNA microarray analysis, a technique which needs roughly 1 μg of total RNA.

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Molecular Autism

RESEARCH Open Access

Exon resequencing of H3K9 methyltransferase complex genes, *EHMT1*, *EHTM2* and *WIZ*, in Japanese autism subjects

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Abstract

Background: Histone H3 methylation at lysine 9 (H3K9) is a conserved epigenetic signal, mediating heterochromatin formation by trimethylation, and transcriptional silencing by dimethylation. Defective GLP (*Ehmt1*) and G9a (*Ehmt2*) histone lysine methyltransferases, involved in mono and dimethylation of H3K9, confer autistic phenotypes and behavioral abnormalities in animal models. Moreover, *EHMT1* loss of function results in Kleefstra syndrome, characterized by severe intellectual disability, developmental delays and psychiatric disorders. We examined the possible role of histone methyltransferases in the etiology of autism spectrum disorders (ASD) and suggest that rare functional variants in these genes that regulate H3K9 methylation may be associated with ASD.

Methods: Since G9a-GLP-Wiz forms a heteromeric methyltransferase complex, all the protein-coding regions and exon/intron boundaries of *EHMT1*, *EHMT2* and *WIZ* were sequenced in Japanese ASD subjects. The detected variants were prioritized based on novelty and functionality. The expression levels of these genes were tested in blood cells and postmortem brain samples from ASD and control subjects. Expression of *EHMT1* and *EHMT2* isoforms were determined by digital PCR.

Results: We identified six nonsynonymous variants: three in *EHMT1*, two in *EHMT2* and one in *WIZ*. Two variants, the *EHMT1* ankyrin repeat domain (Lys968Arg) and *EHMT2* SET domain (Thr961Ile) variants were present exclusively in cases, but showed no statistically significant association with ASD. The *EHMT2* transcript expression was significantly elevated in the peripheral blood cells of ASD when compared with control samples; but not for *EHMT1* and *WIZ*. Gene expression levels of *EHMT1*, *EHMT2* and *WIZ* in Brodmann area (BA) 9, BA21, BA40 and the dorsal raphe nucleus (DoRN) regions from postmortem brain samples showed no significant changes between ASD and control subjects. Nor did expression levels of *EHMT1* and *EHMT2* isoforms in the prefrontal cortex differ significantly between ASD and control groups.

Conclusions: We identified two novel rare missense variants in the *EHMT1* and *EHMT2* genes of ASD patients. We surmise that these variants alone may not be sufficient to exert a significant effect on ASD pathogenesis. The elevated expression of *EHMT2* in the peripheral blood cells may support the notion of a restrictive chromatin state in ASD, similar to schizophrenia.

Keywords: Autism, Rare variant, GLP, G9a, Wiz, Histone methyltransferase, H3K9

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Functional characterization of *FABP3, 5* and *7* gene variants identified in schizophrenia and autism spectrum disorder and mouse behavioral studies

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Disturbances of lipid metabolism have been implicated in psychiatric illnesses. We previously reported an association between the gene for fatty acid binding protein 7 (*FABP7*) and schizophrenia. Furthermore, we identified and reported several rare non-synonymous polymorphisms of the brain-expressed genes *FABP3, FABP5* and *FABP7* from schizophrenia and autism spectrum disorder (ASD), diseases known to part share genetic architecture. Here, we conducted further studies to better understand the contribution these genes make to the pathogenesis of schizophrenia and ASD. In postmortem brains, we detected altered mRNA expression levels of *FABP5* in schizophrenia, and of *FABP7* in ASD and altered *FABP5* in peripheral lymphocytes. Using a patient cohort, comprehensive mutation screening identified six missense and two frameshift variants from the three FABP genes. The two frameshift proteins, FABP3 E132fs and FABP7 N80fs, formed cellular aggregates and were unstable when expressed in cultured cells. The four missense mutants with predicted possible damaging outcomes showed no changes in intracellular localization. Examining ligand binding properties, FABP7 S86G and FABP7 V126L lost their preference for docosahexaenoic acid to linoleic acid. Finally, mice deficient in *Fabp3, Fabp5* and *Fabp7* were evaluated in a systematic behavioral test battery. The *Fabp3* knockout (KO) mice showed decreased social memory and novelty seeking, and *Fabp7* KO mice displayed hyperactive and anxiety-related phenotypes, while *Fabp5* KO mice showed no apparent phenotypes. In conclusion, disturbances in brain-expressed FABPs could represent an underlying disease mechanism in a proportion of schizophrenia and ASD sufferers.

INTRODUCTION

Schizophrenia is a severe mental illness featuring three major symptomatic domains: positive symptoms (e.g. hallucinations and delusions), negative symptoms (e.g. affective flattening and avolition) and cognitive deficits (e.g. disorganized thought). It presents with a life-time prevalence of ~1% worldwide (1). Autism spectrum disorder (ASD) is a relatively common childhood neurodevelopmental disorder, with an increasing incidence. The disease is characterized by severe impairment in

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Research Paper

Zinc finger protein 804A (*ZNF804A*) and verbal deficits in individuals with autism

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Background: In a genome-wide association study of autism, zinc finger protein 804A (*ZNF804A*) single nucleotide polymorphisms (SNPs) were found to be nominally associated in verbally deficient individuals with autism. Zinc finger protein 804A copy number variations (CNVs) have also been observed in individuals with autism. In addition, ZNF804A is known to be involved in theory of mind (ToM) tasks, and ToM deficits are deemed responsible for the communication and social challenges faced by individuals with autism. We hypothesized that *ZNF804A* could be a risk gene for autism. **Methods:** We examined the genetic association and CNVs of *ZNF804A* in 841 families in which 1 or more members had autism. We compared the expression of *ZNF804A* in the postmortem brains of individuals with autism (n = 8) and controls (n = 13). We also assessed in vitro the effect of *ZNF804A* silencing on the expression of several genes known to be involved in verbal efficiency and social cognition. **Results:** We found that rs7603001 was nominally associated with autism (p = 0.018). The association was stronger (p = 0.008) in the families of individuals with autism who were verbally deficient (n = 761 families). We observed *ZNF804A* CNVs in 7 verbally deficient boys with autism. In ZNF804A knockdown cells, the expression of synaptosomal-associated protein, 25kDa (*SNAP25*) was reduced compared with controls (p = 0.009). The expression of *ZNF804A* (p = 0.009) and *SNAP25* (p = 0.009) were reduced in the anterior cingulate gyrus (ACG) of individuals with autism. There was a strong positive correlation between the expression of *ZNF804A* and *SNAP25* in the ACG (p < 0.001). **Limitations:** Study limitations include our small sample size of postmortem brains. **Conclusion:** Our results suggest that *ZNF804A* could be a potential candidate gene mediating the intermediate phenotypes associated with verbal traits in individuals with autism.

Introduction

Autism is a complex neurodevelopmental disorder characterized by deficiencies in social interaction and communication, and by repetitive and stereotyped behaviours. The abnormalities are usually identified in the early years of childhood. Autism is one of the most heritable neurodevelopmental disorders. According to a recent report, the prevalence of this pervasive developmental disorder has risen to 1 in 88. Owing to the genetic heterogeneity and phenotypic variability of autism, classic genetic studies in search of risk genes have not yielded consistent results.

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Although autism has been recognized as a distinct diagnostic entity from schizophrenia, several clinical, biological and genetic overlaps have been observed between these 2 neurodevelopmental disorders. Several psychopathological traits, such as deficits in social interaction and cognition, disruption of emotional processing and sensorimotor gating, and impairments in executive functions, are shared between schizophrenia and autism.[1] Other shared features include abnormalities in brain morphology, neurochemical anomalies and epigenetic risk factors.[1] Whole-genome studies have provided ample evidence for a genetic overlap between these 2 disorders, suggesting common biological pathways in their pathogenesis.[2]

A genome-wide association study (GWAS)[3] and several other independent studies[4–6] have identified zinc finger protein 804A (*ZNF804A*) as the most compelling candidate gene for schizophrenia. Interestingly, in a GWAS of autism, 5 single nucleotide polymorphisms (SNPs) at the *ZNF804A* locus were found to be associated ($p < 0.001$) in verbally deficient individuals with autism (supplementary data of Anney and colleagues, 2010).[7] In addition to the GWAS evidence, copy number variation (CNV) and gene disruption have also been observed at the *ZNF804A* locus (2q32.1) of individuals with autism.[8,9]

ZNF804A has been found to affect neural activation during theory of mind (ToM; also called mentalizing) tasks.[10] Theory of mind is a higher-order form of social cognition representing the ability to infer the mental state of others.[11] It is reported to be impaired in individuals with autism[12] and schizophrenia[13] and is therefore considered as a promising intermediate phenotype for these neurodevelopmental disorders. It is a crucial factor for efficient social interaction.[14] The development of linguistic/verbal abilities and ToM are closely intertwined from infancy.[15] Linguistic abilities have been reported to influence the development of ToM through children's exposure to conversing with people about mental states.[16] Children with linguistic/verbal impairments have been found to perform poorly in verbally dependent ToM tasks.[17] Owing to the presence of a zinc finger domain at its N-terminal end, ZNF804A is deemed to be involved in DNA binding and transcriptional regulation.[18]

On the basis of the previous GWAS[7] linking ZNF804A with verbal deficits in individuals with autism and on the role of ZNF804A in ToM that, in turn, relates to social cognition and verbal skills, we hypothesized that ZNF804A could play a role in predisposing individuals to autism by mediating the intermediate phenotypes associated with verbal traits. We evaluated our hypothesis by conducting a genetic association study of *ZNF804A* with autism, performing a CNV analysis at the *ZNF804A* locus, comparing the expression of *ZNF804A* in the postmortem brains of individuals with autism and healthy controls, and assessing the effect of *ZNF804A* silencing on the expression of genes previously reported to be involved in verbal efficiency and social cognition.

Methods

This study was approved by the Ethics Committee of Hamamatsu University School of Medicine, Hamamatsu, Japan.

Genetic association study

Samples

We obtained DNA samples from the Autism Genetic Resource Exchange (AGRE; www.agre.org).[19] The AGRE has obtained informed consent for the distribution of biological samples to approved researchers. We used DNA samples from 841 families (3211 individuals in total), most of whom were white.

The AGRE website provides pedigree information on each individual along with a diagnosis based on the Autism Diagnostic Interview—Revised (ADI-R).[20] In all, 1467 individuals (1178 male; 289 female) had autism diagnosed based on the ADI-R. Families with a nonidiopathic autism flag (e.g., fragile-X, abnormal brain imaging results, dysmorphic features, birth trauma) recorded for any of its members were not included in the study. Based on the ADI-R score on overall level of language (scores of 0–2), which is an indicator of verbal abilities, individuals with autism were grouped into low verbal (Lvrb; score of 0 or 1) and healthy (Hvrb; score of 2) categories. Verbal deficits were recorded for 1222 individuals with autism belonging to 761 families (Lvrb category).

SNP selection

The genomic structure of *ZNF804A* (positions 185, 171, 338–185, 512, 457 in chromosome 2) is based on the National Center for Biotechnology Innovation B36 human genome assembly (dbSNP b126).

We selected SNPs (MAF > 0.1) from white populations in the International HapMap Project (www.hapmap.org) database. We selected 16 SNPs by aggressive tagging (r^2 threshold = 0.8) using Haploview version 4.1 (www.broad.mit.edu/mpg/haploview). All the SNPs except rs3731834 (missense mutation in exon 4) were located in the introns (see the Appendix, Fig. S1A, available at jpn.ca).

Genotyping

We genotyped the SNPs using the TaqMan method. We purchased Assay-on-Demand TaqMan SNP genotyping assays from Applied Biosystems (ABI). Genotyping polymerase chain reaction (PCR) was carried out in ABI PRISM 7900HT SDS software (ABI) and analyzed using SDS software version 2.0 (ABI).

Statistical analysis

We performed a power analysis using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/dtdt.html). We used FBAT version 2.0.3 (http://biosun1.harvard.edu/~fbat/fbat.htm) to examine the genetic association of *ZNF804A* SNPs with autism in a family-based association test under an additive model. We used the FBAT–MM option for the multimarker test. Statistical analyses were carried out separately for the whole set of 841 families (hereafter referred to as "all families") and for the 761 families with Lvrb children with autism (hereafter referred to as "Lvrb families").

We estimated pairwise linkage disequilibrium (LD) between SNPs, based on the r^2 correlation coefficient, using Haploview. Linkage disequilibrium blocks were defined by the confidence interval algorithm. We examined haplotype

association, and the significance was evaluated by permutation testing (100 000 permutations).

Copy number variation at the ZNF804A *locus*

Copy number variation was examined in the DNA samples of 841 families obtained from AGRE. We analyzed CNV using the TaqMan method in ABI PRISM 7900HT SDS software. The TaqMan CNV assays for *ZNF804A* (Assay ID: Hs00815147_cn; target CNV ID based on the Database of Genomic Variants: Variation_50357) and for the reference gene (telomerase reverse transcriptase [*TERT*]) were purchased from ABI. The CNV analysis of *ZNF804A* and *TERT* were run simultaneously in a duplex real-time PCR. We analyzed 5 ng of each sample in triplicate according to the manufacturer's protocol.

We determined the copy number at the *ZNF804A* locus using CopyCaller software version 2.0 (ABI). The number of copies of the target sequence in each sample was determined by relative quantification using the comparative Ct (ΔΔCt) method, which measures the Ct difference (ΔCt) between target and reference sequences and then compares the ΔCt values of samples to a calibrator sample known to have 2 copies of the target sequence. The copy number of the target is estimated to be 2 times the relative quantity.

ZNF804A *silencing*

The expression of *ZNF804A* was found to be low in the commonly used cell lines, such as HEK 293 and SK-N-SH, whereas a robust expression was observed in SH-SY5Y human neuroblastoma cell line (data not shown). We therefore examined the effect of *ZNF804A* silencing in SH-SY5Y cell lines.

The expression of *ZNF804A* was knocked down in SH-SY5Y cells by RNA interference (RNAi) using gene-specific small interfering RNAs (siRNAs). Sufficient gene silencing could not be achieved using the routine methods of transfection (Lipofectamine 2000, FuGENE HD, Accell SMARTpool siRNA). Efficient silencing of *ZNF804A* was achieved by electroporation using the Neon Transfection System (Invitrogen). Electroporation was performed according to the manufacturer's instructions. Briefly, 2×10^5 cells (5 replicates each for *ZNF804A* RNAi and negative control RNAi) were suspended in 10 μL electroporation buffer containing either 100 nM *ZNF804A* siRNA (ID: s40770; Ambion) or 100 nM negative control siRNA (Negative Control #1 siRNA; Ambion) and electroporated (1500 V, 20 ms, 1 pulse) in 10 μL tips. The cells (10 μL electroporated cells in 2 mL medium [Ham's F12 and Eagle's minimum essential medium in 1:1 ratio, supplemented with 2 mM glutamine, 1% nonessential amino acids and 15% fetal bovine serum]) were grown (37°C; 5% CO_2) in 6-well plates for 72 hours.

Extraction of RNA

We extracted total RNA from SH-SY5Y cells using TRIzol Reagent (Invitrogen) in accordance with the manufacturer's protocol. The RNA samples were further purified using RNeasy Micro Kit (QIAGEN GmbH); this protocol includes a DNase treatment step. The quantity (absorbance at 260 nm) and quality (ratio of absorbance at 260 nm and 280 nm) of RNA were estimated with a NanoDrop ND-1000 Spectrophotometer (Scrum).

Real-time quantitative PCR

We synthesized complementary DNA (cDNA) from total RNA using the ImProm-II Reverse Transcription System (Promega) following the manufacturer's protocol for oligo (dT) primer.

We performed quantitative PCR (qPCR) analysis using the TaqMan method in ABI PRISM 7900HT SDS software. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous reference. TaqMan assays for *ZNF804A* (Hs00290118_s1) and *GAPDH* (Pre-developed TaqMan Assay Reagent) were purchased from ABI. Each assay was performed in triplicate. Cycle threshold (Ct) values of the target gene were normalized (ΔCt) to that of *GAPDH* (ΔCt = target gene Ct – *GAPDH* Ct). Any alteration in gene expression in the *ZNF804A*-silenced cells was analyzed by relative quantification (ΔΔCt) against the negative control cells (ΔΔCt = ΔCt of *ZNF804A* RNAi – ΔCt of negative control). We determined the fold-change in gene expression between the 2 groups of cells by calculating $2^{-\Delta\Delta Ct}$. Any difference in *ZNF804A* expression between the 2 groups of cells was evaluated using the *t* test.

Further, the expression of the following genes, previously reported to be associated with verbal/linguistic abilities and social cognition, was compared between *ZNF804A*-silenced cells and negative control cells by SYBR Green qPCR: *BDNF*,[21] *CNTNAP2*,[22] *DISC1*,[23] *DRD2*,[24] *FOXP2*,[25] *NRG1*,[26] *OXTR*,[27] *SHANK3*,[28] *SNAP25*,[29] *SRPX2*[30] and *TCF4*.[31] We designed qPCR primers (see the Appendix, Table S1) using Primer Express version 2.0 (ABI). The efficiency of these primers ranged between 0.93 and 1.03. The specificity of amplicons was demonstrated by melting curve analysis (single peak at 83–86°C).

We used the QuantiTect SYBR Green PCR kit (QIAGEN) for qPCR assays; each assay was carried out in triplicate. We used *GAPDH* as the reference gene. The qPCR analysis was performed in ABI PRISM 7900HT SDS software. Any alteration in gene expression between the 2 groups of cells was estimated by the relative quantification method described earlier. We evaluated the difference in gene expression between *ZNF804A* silenced cells and negative control cells using a *t* test, and any correlation between the expression of *ZNF804A* and other genes was examined using the Pearson correlation coefficient.

Western blot confirmation of ZNF804A silencing

The protein expression of ZNF804A and SNAP25 in ZNF804A-silenced SH-SY5Y cells and negative control siRNA-transfected cells were compared using Western blot. The cells were homogenized in radioimmunoprecipitation assay buffer. The total protein in the lysate was quantified using Pierce bicinchoninic acid assay kit (Thermo Scientific). We separated 10 μg of each sample on 10% SDS/polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto a polyvinylidene fluoride membrane (Millipore), blocked and incubated with the primary antibody at 4°C overnight. The following primary antibodies

were used: anti-ZNF804A (Santa Cruz Biotechnology) at 1:200 dilution for the detection of ZNF804A, anti-SNAP25 (Abcam) at 1:500 dilution for the detection of SNAP25 and anti-GAPDH (Abcam) at 1:5000 dilution for the detection of GAPDH, which was used as the loading control. The blots were then washed, incubated with 1:15 000 diluted IRDye-conjugated secondary antibody (Rockland) for 1 hour and washed again. The blots were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Gene expression in postmortem brain samples

Postmortem brain tissues

Postmortem brain samples from individuals with autism and healthy controls were provided by the Autism Tissue Program (ATP; www.autismtissueprogram.org), National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders (NICHD BTB; http://medschool.umaryland.edu/btbank/) and the Harvard Brain Tissue Resource Center (www.brainbank.mclean.org/). Frozen tissue samples from the anterior cingulate gyrus (ACG), motor cortex (MC) and thalamus were used in the study.

Extraction of RNA

The brain tissues (~75 mg obtained by macrodissection) were homogenized by ultrasonication, and total RNA was extracted using TRIzol Reagent (Invitrogen). We performed RNA purification and quantification as described previously.

Quantitative PCR

We performed cDNA synthesis as described previously. The expression of *ZNF804A* and synaptosomal-associated protein,

Table 1: Family-based association test analysis of *ZNF804A* with autism

				Families†		Frequency		*p* value‡	
SNP	Physical position	Allele*	Location	All§	Lvrb§	All§	Lvrb§	All§	Lvrb§
rs13393273	185185922	A	Intron 1	591	532	0.619	0.618	0.24	0.08
		G				0.381	0.382		
rs12613195	185197466	C	Intron 1	551	499	0.682	0.679	0.57	0.59
		G				0.318	0.321		
rs12693385	185215474	T	Intron 1	604	548	0.520	0.518	0.60	0.40
		C				0.480	0.482		
rs990844	185227330	T	Intron 1	323	287	0.867	0.869	0.24	0.10
		G				0.133	0.131		
rs7597593	185241825	C	Intron 1	617	549	0.600	0.602	0.50	0.22
		T				0.400	0.398		
rs1038197	185265516	A	Intron 1	480	429	0.760	0.764	0.11	0.08
		G				0.240	0.236		
rs13026742	185313227	C	Intron 1	597	536	0.579	0.579	0.18	0.25
		T				0.421	0.421		
rs1987025	185355840	T	Intron 1	479	425	0.750	0.746	0.12	0.09
		A				0.250	0.254		
rs17509608	185440823	C	Intron 2	295	270	0.892	0.892	0.74	> 0.99
		T				0.108	0.108		
rs7603001	185475061	G	Intron 2	596	539	0.510	0.506	0.018	0.008
		A				0.490	0.494		
rs1344706	185486673	T	Intron 2	584	524	0.637	0.635	0.16	0.13
		G				0.363	0.365		
rs7593816	185490557	C	Intron 2	412	375	0.809	0.806	0.59	0.45
		T				0.191	0.194		
rs3731834	185511609	C	Exon 4 (L/V)	388	349	0.830	0.833	0.29	0.48
		G				0.170	0.167		
rs10931157	185513698	A	3'	542	484	0.704	0.702	0.21	0.06
		G				0.296	0.298		
rs12693402	185516324	C	3'	396	351	0.822	0.826	0.20	0.07
		T				0.178	0.174		
rs4380187	185520185	A	3'	616	554	0.570	0.567	0.50	0.46
		C				0.430	0.433		

L/V = leucine/valine; Lvrb = autistic, low verbal; SNP = single nucleotide polymorphism; *ZNF804A* = zinc finger protein 804A.
*Major allele is listed first.
†No. of informative families used.
‡$p < 0.05$, additive model.
§Whole set of 841 pedigrees; Lvrb: 761 pedigrees.

25kDa (*SNAP25*) were compared in the postmortem brains of individuals with autism and healthy controls. We performed qPCR analysis using the TaqMan method in ABI PRISM 7900HT SDS software. We used *GAPDH* as the endogenous reference. The Ct values of the target gene were normalized (ΔCt) to that of *GAPDH*. Any alteration in gene expression in the autism group was analyzed by relative quantification (ΔΔCt) against the control group. We determined the fold change in gene expression between the autism and control groups by calculating $2^{-\Delta\Delta Ct}$.

Statistical analysis

We examined the difference in age, postmortem interval (PMI) and gene expression between the autism and control groups using a *t* test, and the χ^2 test was used to examine the difference in sex distribution between the 2 groups. Any correlation between the expression of *ZNF804A* and *SNAP25* was examined using the Pearson correlation coefficient.

Results

Genetic association study

Power analysis showed that the overall sample size of 841 families provides 91% power to detect an odds ratio of 1.5 for an allele frequency of 0.1 at an α of 0.05.

In the family-based association test (Table 1), rs7603001 located in intron 2 of *ZNF804A* was nominally associated with autism (z score for risk allele A = 2.362, $p = 0.018$). When individuals with autism were categorized based on verbal abilities, a stronger association of this SNP was found in the Lvrb families (z score for risk allele A = 2.657, $p = 0.008$), whereas no association was observed in the Hvrb families (z score = 0, $p > 0.99$; data not shown). The A allele of rs7603001 was overtransmitted to the individuals with autism (transmission 53% in all families v. 54% in Lvrb families). The genetic association, however, did not withstand multiple testing correction. None of the other SNPs showed any significant association with autism. Genotypic distribution of SNPs were in Hardy–Weinberg equilibrium.

Three LD blocks were identified in *ZNF804A* (Table 2; Appendix, Fig. S1B). The haplotype ACTCATC in the second LD block (rs1038197, rs13026742, rs1987025, rs17509608, rs7603001, rs1344706, rs7593816) showed a significant association with autism in the Lvrb families (z score = 3.103, $p = 0.004$). This haplotype includes the risk allele A of rs7603001. The association remained significant ($p = 0.047$) following multiple testing correction by permutation analysis (100 000 permutations). Interestingly, the haplotype ACTCGTC that includes the protective G allele of rs7603001 showed a tendency toward association with autism in the Lvrb families (z score = –1.907, $p = 0.05$).

Taken together, the A allele of rs7603001 may be considered as a risk allele and the G allele as a protective allele of autism in individuals with verbal defects.

Copy number variation at the ZNF804A *locus*

We observed CNV at the *ZNF804A* locus in the same DNA samples that we used in our genetic association study (Table 3): copy number gain (3 copies) in 6 samples and copy number loss (1 copy) in 2 samples. One of the CNVs (gain)

Table 2: Haplotype association analysis of *ZNF804A* with autism in the low verbal subgroup

Block; haplotype	Frequency	*p* value
Block 1 (SNPs 01–04)		
GCTT	0.377	0.09
AGCT	0.317	0.57
ACCT	0.16	0.06
ACTG	0.135	0.09
Block 2 (SNPs 06–12)		
GTACATC	0.234	0.08
ACTCGGT	0.193	0.69
ACTCGGC	0.178	0.13
ACTCGTC	0.143	0.05
ATTTATC	0.104	0.57
ATTCATC	0.073	0.54
ACTCATC	0.057	0.004
Block 3 (SNPs 14,15)		
AC	0.531	0.73
GC	0.292	0.07
AT	0.177	0.08

SNP = single nucleotide polymorphism; *ZNF804A* = zinc finger protein 804A.

Table 3: Copy number variation at *ZNF804A* locus

Sample ID*	Sex	Age, yr	Affection status	CNV	Gain/loss	De novo/inherited	Lvrb/Hvrb
AU0154302	Male	14	Autism	3	Gain	De novo	Lvrb
AU023803	Male	8	Autism	3	Gain	De novo	Lvrb
AU077304	Male	16	Autism	3	Gain	De novo	Lvrb
AU0871302	Male	7	Autism	1	Loss	De novo	Hvrb
AU1092302	Male	3	Autism	3	Gain	Inherited	Lvrb
AU1466302	Male	10	Autism	1	Loss	De novo	Lvrb
AU1650305	Male	7	Autism	3	Gain	De novo	Lvrb
AU1655301	Male	16	Autism	3	Gain	De novo	Lvrb

CNV = copy number variation; Hvrb = autistic, healthy; Lvrb: autistic, low verbal; *ZNF804A* = zinc finger protein 804A.
*Autism Genetic Resource Exchange (AGRE) identifier.

was inherited from the mother, whereas the other CNVs were caused by de novo events. All the CNVs were observed in boys with autism (age 7–16 yr); all but 1 of them belonged to the Lvrb category. We also observed CNVs in 7 maternal samples (gain in 6 and loss in 1 sample) and in 2 paternal samples (gain in 1 and loss in 1 sample).

ZNF804A *silencing*

Figure 1A shows a significant difference in the expression of *ZNF804A* between the cells electroporated with *ZNF804A*-specific siRNA and the negative control ($p = 0.003$). In qPCR, the expression of *ZNF804A* was knocked down by 77%. *ZNF804A* silencing was confirmed by Western blot (Fig. 1B).

In the *ZNF804A*-knockdown SH-SY5Y cells, the expression of *SNAP25* was significantly reduced compared with the negative controls ($p = 0.009$; Fig. 1C). This was confirmed by Western blot (Fig. 1B). We also found a significant positive correlation between the expression of *ZNF804A* and *SNAP25* (Pearson $r = 0.713$, $p = 0.006$; Fig. 1D).

There was no significant alteration in the expression of other genes (data not shown).

Gene expression in postmortem brain

We obtained postmortem brain samples from the ACG (8 autism, 13 control), MC (7 autism, 8 control) and thalamus (8 autism, 9 control). Demographic characteristics of the individuals from whom the samples were obtained are described in Table 4.

There was no significant difference in age, postmortem interval and sex distribution between the control and autism groups (see the Appendix, Table S2). The expression of *ZNF804A* (fold-change $2^{-\Delta\Delta Ct} = 0.277$, $p = 0.009$) and *SNAP25* ($2^{-\Delta\Delta Ct} = 0.258$, $p = 0.009$) were significantly reduced in the ACG of individuals with autism compared with controls (Fig. 2A and B). We also found a strong positive correlation between the expression of *ZNF804A* and *SNAP25* in the ACG (Pearson $r = 0.837$, $p < 0.001$; Fig. 2C). In the MC and thalamus, the expression of *ZNF804A* or *SNAP25* did not differ

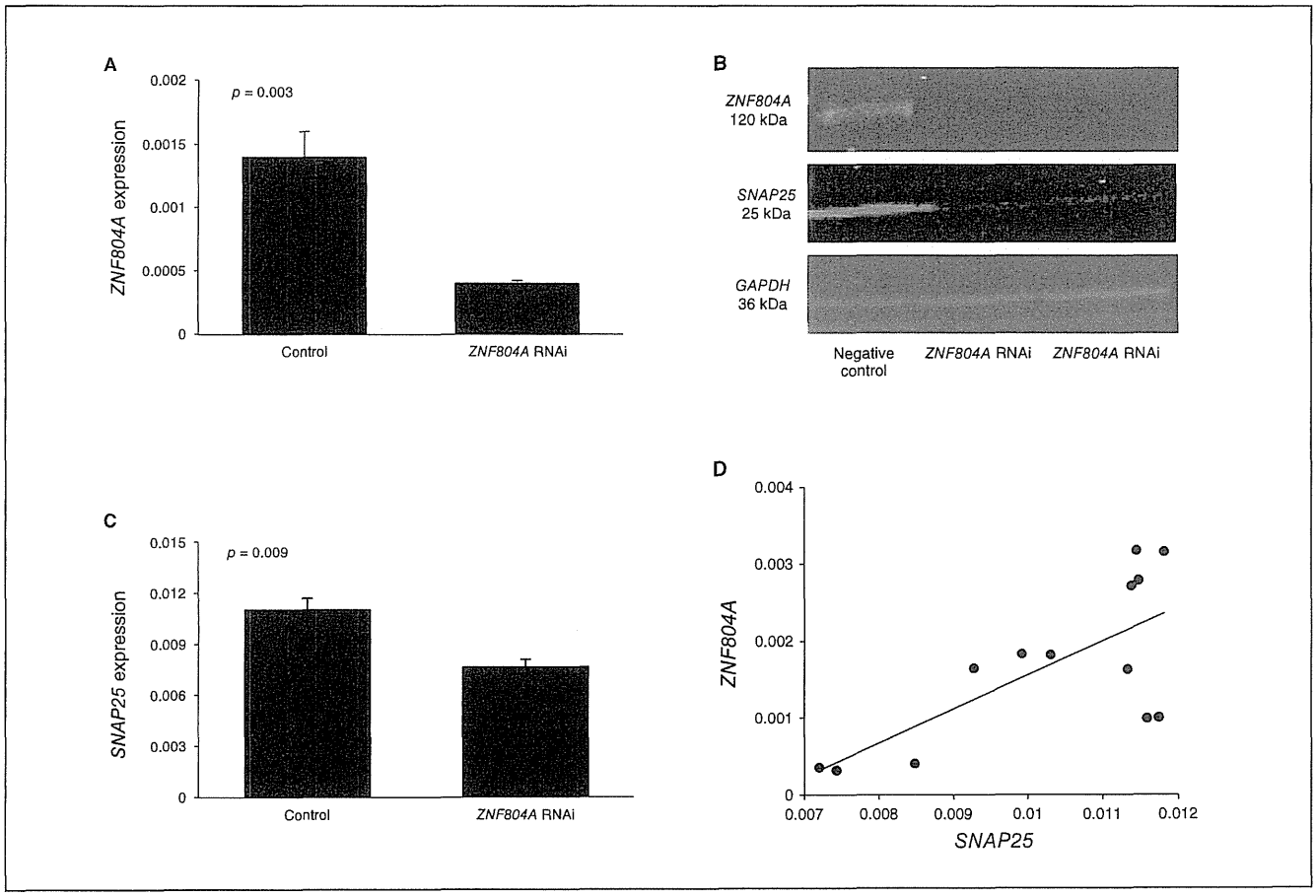

Fig. 1: Zinc finger protein 804A (*ZNF804A*) silencing in SH-SY5Y cells. **(A)** *ZNF804A* expression was knocked down by 77% ($p = 0.003$) in the SH-SY5Y cells electroporated with *ZNF804A*-specific small interfering RNA (siRNA) compared with the negative controls. **(B)** Comparison of the expression of *ZNF804A* and *SNAP25* between *ZNF804A*-silenced SH-SY5Y cells and negative control siRNA-transfected SH-SY5Y cells in Western blot. The expression of *SNAP25* was downregulated in *ZNF804A*-silenced cells. *GAPDH* was used as the loading control. **(C)** *SNAP25* expression was significantly lower in the *ZNF804A*-silenced cells compared with the negative controls ($p = 0.009$). **(D)** Positive correlation between the expression of *ZNF804A* and *SNAP25* in SH-SY5Y cells (Pearson $r = 0.713$; $p = 0.006$).

significantly between the control and autism groups (data not shown).

Discussion

We suggest that *ZNF804A* could be a risk gene mediating the intermediate phenotypes related to verbal skills in individuals with autism. In a GWAS of autism, Anney and colleagues (supplementary data)[7] reported nominal association of several *ZNF804A* SNPs (rs17508877, rs1038197, rs7585738, rs6730122, rs10199843) with the Lvrb subset of individuals with autism. To our knowledge, the present study is the first to confirm the association of *ZNF804A* with a subgroup of individuals with autism characterized by verbal deficits.

The SNP rs7603001, which showed nominal association with autism in all families and in the subset of Lvrb families, is located in intron 2 of *ZNF804A*. Even though this SNP may not have a functional significance, putative regulatory regions have been predicted (FastSNP; http://fastsnp.ibms.sinica.edu.tw/pages/inputSNPListAnalysis.jsp) for the SNPs

Table 4: Postmortem brain tissue information

Sample ID*	Diagnosis	Age, yr	Sex	PMI, h	Race	Cause of death	Brain region†
818	Control	27	M	10	White	Multiple injuries	ACG
1065	Control	15	M	12	White	Multiple injuries	ACG, THL
1297	Control	15	M	16	African American	Multiple injuries	ACG, MC, THL
1407	Control	9	F	20	African American	Asthma	ACG, MC, THL
1541	Control	20	F	19	White	Head injuries	ACG, MC, THL
1649	Control	20	M	22	Hispanic	Multiple injuries	ACG, MC, THL
1708	Control	8	F	20	African American	Asphyxia, multiple injuries	ACG, MC, THL
1790	Control	13	M	18	White	Multiple injuries	ACG
1793	Control	11	M	19	African American	Drowning	ACG, MC, THL
1860	Control	8	M	5	White	Cardiac arrhythmia	ACG
4543	Control	28	M	13	White	Multiple injuries	ACG, MC, THL
4638	Control	15	F	5	White	Chest injuries	ACG
4722	Control	14	M	16	White	Multiple injuries	ACG, MC, THL
797	Autism	9	M	13	White	Drowning	ACG, THL
1638	Autism	20	F	50	White	Seizure	ACG, MC, THL
4231	Autism	8	M	12	African American	Drowning	ACG, MC, THL
4721	Autism	8	M	16	African American	Drowning	ACG, MC, THL
4899	Autism	14	M	9	White	Drowning	ACG, MC, THL
5000	Autism	27	M	8.3	NA	NA	ACG, MC, THL
6294	Autism	16	M	NA	NA	NA	ACG, MC, THL
6640	Autism	29	F	17.83	NA	NA	ACG, MC, THL

ACG = anterior cingulate gyrus; F = female; M = male; MC = motor cortex; NA = not available; PMI = postmortem interval; THL = thalamus.
*Autism Tissue Program (ATP) identifier.
†Brain regions for which each sample was available.

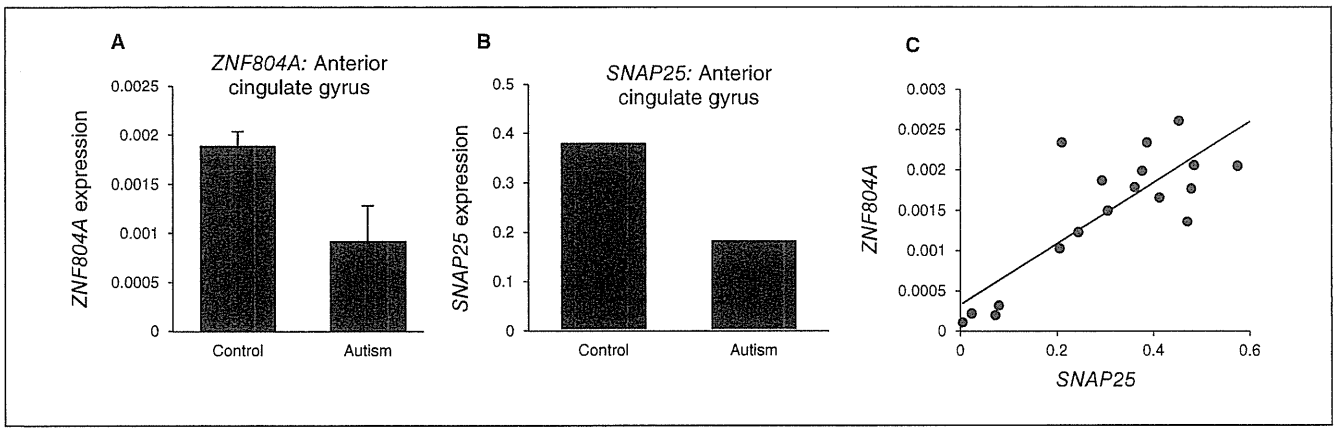

Fig. 2: Gene expression in postmortem brain. The expression of **(A)** zinc finger protein 804A (*ZNF804A*; $p = 0.009$) and **(B)** *SNAP25* ($p = 0.009$) were significantly reduced in the anterior cingulate gyrus (ACG) of individuals with autism compared with healthy controls. **(C)** Positive correlation between the expression of *ZNF804A* and *SNAP25* in the ACG (Pearson $r = 0.837$; $p < 0.001$).