Hum Genet (2006) 119: 558–570
DOI 10.1007/s00439-006-0172-7

ORIGINAL INVESTIGATION

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Genome scans and gene expression microarrays converge to identify gene regulatory loci relevant in schizophrenia

Received: 4 October 2005 / Accepted: 14 March 2006 / Published online: 8 April 2006

Abstract Multiple linkage regions have been reported in schizophrenia, and some appear to harbor susceptibility genes that are differentially expressed in postmortem brain tissue derived from unrelated individuals. We combined traditional genome-wide linkage analysis in a multiplex family with lymphocytic genome-wide expression analysis. A genome scan suggested linkage to a chromosome 4q marker (D4S1530, LOD 2.17, $\theta = 0$) using a dominant model. Haplotype analysis using flanking microsatellite markers delineated a 14 Mb region that cosegregated with all those affected. Subsequent genome-wide scan with SNP genotypes supported the evidence of linkage to 4q33–35.1 (LOD = 2.39) using a dominant model. Genome-wide microarray analysis of five affected and five unaffected family members identified two differentially expressed genes within the haplotype *AGA* and *GALNT7* (aspartylglucosaminidase and UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 7) with nominal significance; however, these genes did not remain significant following analysis of covariance. We carried out genome-wide linkage analyses between the quantitative expression phenotype and genetic markers. *AGA* expression levels showed suggestive linkage to multiple markers in the haplotype (maximum LOD = 2.37) but to no other genomic region. *GALNT7* expression levels showed linkage to regulatory loci at 4q28.1 (maximum LOD = 3.15) and in the haplotype region at 4q33–35.1 (maximum LOD = 2.37). *ADH1B* (alcohol dehydrogenase IB) was linked to loci at 4q21–q23 (maximum LOD = 3.08) and haplotype region at 4q33–35.1 (maximum LOD = 2.27). Seven differentially expressed genes were validated with RT-PCR. Three genes in the 4q33–35.1 haplotype region were also differentially expressed in schizophrenia in postmortem dorsolateral prefrontal cortex: *AGA*, *HMGB2*, and *SCRG1*. These results indicate that combining differential gene expression with linkage analysis may help in identifying candidate genes and potential regulatory sites. Moreover, they also replicate recent findings of complex *trans*- and *cis*- regulation of genes.

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Introduction

Genome-wide linkage analysis is an important tool for mapping complex genetic disorders such as schizophrenia. While linkage has been predictably more successful for Mendelian disorders, linkage analysis has identified a number of complex disease loci. To date, multiple linkage regions have been reported in schizophrenia, and some appear to harbor schizophrenia susceptibility genes (O'Donovan et al. 2003; Harrison and Owen 2003). With the exception of a few genes, such as neuregulin 1 (Stefansson et al. 2002) and dysbindin (Straub et al. 2002), there have been no other replicated genes found to be associated with schizophrenia in linkage regions. With a sufficient number of informative meioses (~100 to 200) linkage can narrow a disease gene search for a Mendelian-based disorder to an interval of ~1 cM. For complex diseases, due to incomplete penetrance and the inability to distinguish phenocopies from true recombination events, typically linkage can only narrow a disease gene search to an area of 10–30 cM. Such regions contain hundreds of potential candidate genes. Additional methods are needed to help prioritize the

selection of candidate genes for mutation searches in broad linkage regions. It is known that noncoding variants can alter gene transcription (Knight 2005). Thus, genes that are differentially expressed in linkage regions may be attractive candidates for mutation searches and postmortem expression studies.

While access to brain tissue is not possible for living subjects, a readily available and commonly used source of mRNA for expression studies are lymphocytes, due to their availability from living participants. Lymphocyte tissue is of a different embryological origin than brain and does not express all brain relevant genes, while lymphocytes do show abundant expression of genes found in neural tissues (M. P. Vawter, unpublished results). Another advantage of lymphocyte gene expression is the relative absence of confounding factors, such as postmortem interval, agonal factor, or pH (Vawter et al. 2004; Tsuang et al. 2005). Medications can affect in vivo gene expression, but these effects might be mitigated in transformed lymphocytes utilized in the present study. Moreover, regulatory mutations that alter gene expression in the brain may lead to altered expression in other tissues. It is now possible to study gene expression on a genome-wide basis using high-density microarrays, and such investigations may help identify genes underlying illness within linkage regions. In addition, microarray investigation of gene expression also makes it possible to study whether a gene (or genes) within a linkage region is coregulated with transcription of other genes in the genome. Such studies may identify other loci important for schizophrenia as well as regulatory or metabolic pathways underlying the pathophysiology. We have combined genome linkage analysis and genome-wide expression in the present study.

Methods

Subjects

All subjects provided informed consent for genetic mapping studies, and the institutional review board approved all protocols. The multiplex pedigree consisted of 20 members (Fig. 1). Seventeen individuals in the pedigree were initially scanned with microsatellite markers for genome-wide linkage analysis. Ten informative subjects were selected for final screening: five individuals with schizophrenia and the haplotype, and five unaffected controls without the haplotype (Table 1). These 10 subjects (Fig. 1) were analyzed by microarray, SNP microarray genotyping, Q-PCR, and mutation scanning.

RNA and DNA isolation from lymphocytes

The procedure for generating cell lines and extraction of total RNA was followed as previously described (Vawter et al. 2004). RNA was extracted from $\sim 5\times10^7$ lymphoblastic cells using the standard TRIzol isolation protocol (Invitrogen, Carlsbad, CA, USA). The total RNA was cleaned by passing over silica-based mini-spin columns (Qiagen RNeasy Mini Kit, Valencia, CA, USA) and analyzed on a 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) for quantification of 28 and 18S ribosomal RNA peaks. One flask of 5×10^7 cells was used for genomic DNA extraction using the standard chloroform/isoamyl alcohol isolation procedure. DNA was precipitated from the aqueous layer using a final concentration of 0.3 M sodium acetate and 1.5 volumes of ice-cold 95% isopropyl alcohol, and the precipitate was collected by centrifugation. DNA was resuspended in 1 ml of TE, and the purity and concentration were determined by spectrophotometric absorbance at 260 and 280 nm (BioRad SmartSpec 3000).

Postmortem DLPFC microarray study

A microarray study of dorsolateral prefrontal cortex (DLPFC) from the Stanley Foundation Microarray collection used 34 subjects with bipolar disorder, 36 subjects with schizophrenia, and 36 controls was run on the Codelink 20 K platform (GE Health, AZ, USA). The demographics of the subjects for each group are shown (Table 3), the study is being reported separately (M. P. Vawter, unpublished results). The Codelink 20 K platform uses a 30-mer probe, and fluorescent glass slides, to interrogate approximately 19,300 human Genbank accessions from cDNA, RefSeq mRNA, and Unigene clusters. The labeling, hybridization, and

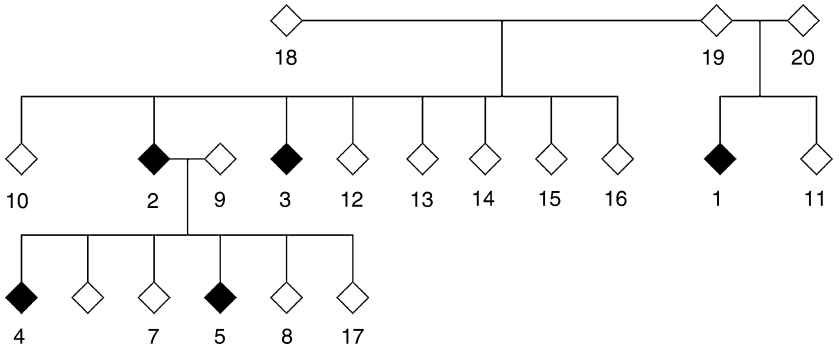

Fig. 1 Multiplex-pedigree showing schizophrenia (*black diamond*) and unaffected family members (*open diamond*). Individuals 1–10 were studied by microarray, SNP genotype, and Q-PCR. Individuals 1–17 were included in the genome-wide microsatellite marker linkage scan

Table 1 Demographics for each subject used in microarray gene expression, Q-PCR, and SNP genotype studies

Group	Age (years)	Gender	Age of onset	RNA 28S/18S	Affymetrix microarray U133		
					Present calls	3′/5′ GAPDH	3′/5′ β-actin
Schizophrenia							
A	60	F	32	1.9	45.4	0.94	1.13
A	48	F	18	2.0	43.5	0.94	1.05
A	42	M	19	1.3	46.0	0.92	1.15
A	24	M	20	1.2	46.7	1.02	1.35
A	18	M	17	1.4	45.4	0.95	1.21
Average	38.4	2F/3M	21.2	1.6	45.4	1.0	1.2
Standard deviation	17.3		6.1	0.3	1.2	0.04	0.1
Control							
U	22	F	NA	1.6	43.8	1.68	2.73
U	21	F	NA	1.4	45.3	1.03	1.34
U	16	M	NA	1.4	46.2	1.02	1.39
U	48	M	NA	2.1	40.7	1.87	4.11
U	34	F	NA	1.4	43.5	0.95	1.18
Average	28.2	3F/2M		1.6	43.9	1.3	2.2
Standard deviation	12.9			0.3	2.1	0.4	1.3
Schizophrenia–control (*t* test *P* value)	0.3			0.9	0.2	0.1	0.2

scanning of the microarrays were followed using the manufacturer's protocol (GE Health). The expression of genes that were located in the 4q33–35.1 region are shown for controls, bipolar disorder, and schizophrenia (Table 4).

Genome scan with microsatellite markers

An initial genome scan of nine families, each containing three to five cases of schizophrenia, was previously conducted with 329 polymorphic DNA markers spaced at approximately 15–20 cM intervals as described (Coon et al. 1994). We further analyzed in the present study one pedigree with 5 affected and 12 unaffected individuals that showed suggestive linkage on chromosome 4q. Fine mapping was completed with microsatellite markers spaced at approximately 2 cM intervals. Radiolabeled PCR-products were separated on a 6% denaturing polyacrylamide gel followed by autoradiography. Two technicians blind to diagnosis scored genotypes independently.

GeneChip mapping assay

A linkage scan of this multiplex pedigree was performed using the GeneChip® Mapping10 K Xba Array containing 11,529 SNP markers (Affymetrix Inc., Santa Clara, CA, USA). Briefly, 250 ng of genomic DNA was digested with the restriction endonuclease XbaI (0.5 U/μl) for 2 h in the Applied Biosystems 9700 GeneAmp PCR System. The digested DNA was ligated using 0.25 μM Adaptor Xba and 1× T4 DNA ligase for 2 h. Ligated DNA was amplified by PCR in four separate reactions using 0.75 μM Xba PCR Primer and 0.1 U/μl AmpliTaq Gold polymerase. Each PCR product was run on a 2% TBE agarose gel to ensure successful amplification. The PCR products were pooled and cleaned using Qiagen QIAquick columns and DNA concentration determined by spectrophotometric absorbance at 260 nm (BioRad SmartSpec 3000). The purified samples were fragmented with 0.048 U/μl Fragmentation Reagent (Affymetrix) for 30 min. To check for proper fragmentation, 4 μl of fragmented PCR was run on a 4% TBE agarose gel. Fragments were end-labeled using 0.143 mm GeneChip DNA labeling reagent and 1.5 U/μl terminal deoxynucleotidyl transferase for 2 h. The labeled DNA was hybridized to a GeneChip® Mapping10 K array at 48°C for 16–18 h at 60 rpm in the Affymetrix 640 hybridization oven. After hybridization, the arrays were washed, stained, and scanned using an Affymetrix Fluidics Station F450 with images obtained by the Affymetrix GeneArray® scanner 3000. Affymetrix MicroArray Suite software was used to generate raw microarray feature intensities (RAS scores). RAS scores were processed using Affymetrix Genotyping Tools software GCOS/GDAS (Affymetrix Inc.) to derive SNP genotypes, marker order, and linear chromosomal location.

The SNP genotype data were formatted for dCHIP SNPLinkage software (Leykin et al. 2005). There were a total of 11,229 SNPs genotyped with a 90% average SNP detection rate across 10 arrays (range 85–95%). Of 11,229 SNPs on the Affymetrix SNP chip that were genotyped, 3,235 SNPs were not informative mainly due to homozygosity in all family members. A small fraction (160) were omitted due to Mendelian errors in the pedigree leaving 7,994 SNP markers for linkage analysis.

Linkage analysis

Parametric analysis of microsatellite markers to the disease assumed a dominant model, (θ recursively

Table 2 Linkage of SNP genotype markers to schizophrenia in pedigree shown in Fig. 1 using dChip Linkage. Similar linkage results were obtained with MERLIN parametric dominant linkage testing

CHR	NCBI	cM	LOD	dbSNP RS ID
4	167.973	161.3	−0.3	rs1246507
4	169.018	162	−0.31	rs2319145
4	170.007	162.9	−0.3	rs497179
4	170.099	163.1	−0.3	rs1387330
4	170.534	163.7	−0.31	rs1318822
4	171.983	165.3	−0.31	rs1403225
4	172.293	165.6	−0.3	rs402841
4	172.945	166.2	0.36	rs2332211
4	172.963	166.2	0.36	rs958355
4	172.963	166.2	0.36	rs958357
4	172.963	166.2	0.36	rs1038813
4	173.079	166.3	0.41	rs952008
4	173.171	166.4	0.46	rs3907555
4	173.625	166.9	0.64	SNP_A-1508870
4	173.625	166.9	0.64	SNP_A-1509404
4	173.629	166.9	0.64	rs718913
4	173.968	167.3	0.74	rs1812424
4	174.091	167.4	1.54	rs2200257
4	174.511	167.6	1.96	rs1347703
4	174.587	167.7	2.08	rs1370576
4	174.587	167.7	2.08	rs1370577
4	174.893	167.9	2.25	rs3905287
4	175.367	168.1	2.37	rs2119788
4	176.767	170	2.38	rs985261
4	177.437	170.3	2.39	rs2333244
4	177.454	170.3	2.39	rs727917
4	178.123	171.4	2.39	rs723818
4	178.123	171.4	2.39	rs723819
4	178.123	171.4	2.39	rs723820
4	178.262	171.6	2.39	rs309753
4	178.326	171.7	2.39	rs404409
4	178.326	171.7	2.39	rs448261
4	178.343	171.7	2.39	rs309773
4	178.349	171.7	2.39	rs1375749
4	178.516	171.9	2.39	rs1485765
4	179.029	172.5	2.39	rs1821970
4	179.029	172.5	2.39	rs1371231
4	179.029	172.5	2.39	rs722832
4	179.206	172.8	2.39	rs1395477
4	179.575	173.2	2.39	rs1902018
4	179.589	173.2	2.39	rs1454214
4	179.858	173.7	2.39	rs966108
4	180.001	174	2.39	rs722387
4	180.101	174.1	2.39	rs1397413
4	180.504	174.9	2.39	rs727353
4	180.569	175	2.39	rs720096
4	180.574	175	2.39	rs1368870
4	180.574	175	2.39	rs1368871
4	180.9	175.6	2.39	rs4103290
4	181.438	176.6	2.39	rs1455691
4	181.68	177.2	2.39	rs1379996
4	181.978	177.8	2.39	rs1112857
4	181.978	177.8	2.39	rs6815286
4	181.996	177.8	2.39	rs1343863
4	182.35	178.4	2.38	rs2309248
4	182.859	179.8	2.36	rs1969398
4	182.86	179.8	2.36	rs1879908
4	183.346	180.6	2.35	rs724658
4	183.346	180.6	2.35	rs724659
4	183.638	181	2.14	rs726856
4	183.799	181.3	1.85	rs1914369
4	183.882	181.6	0.69	SNP_A-1519703
4	184.275	183.1	−0.43	rs955638
4	185.301	186	−2.36	rs1113122
4	185.301	186	−2.36	rs868082
4	186.462	190.3	−3.26	rs1077767
4	187.227	192.9	−4.28	rs2310160
4	187.583	193.6	−4.39	rs726466
4	187.888	194.9	−4.63	rs1378149

varied from 0.05–0.45) and was calculated using LINKAGE software. dChip SNP Linkage and MERLIN (Abecasis et al. 2002) were both used to analyze linkage of phenotype to SNP genotype data. SNP data for autosomal chromosomes were used for linkage analysis in the pedigree by a variant of the Lander–Green algorithm in the dChip Linkage module of dChip to perform multipoint parametric linkage analysis and compute a LOD score at each SNP position (Lander and Green 1987; Kruglyak et al. 1996, 1995). We verified linkage found with dChip Linkage with MERLIN version 1.0.1 software. The MERLIN software package version 1.0.1 (Abecasis et al. 2002) was used to analyze linkage to the disease with a dominant parametric model for both SNP and MSM markers. In using either MERLIN or dChip Linkage, there was only one suggestive linkage region found in the genome scan at chromosome 4q.

MERLIN was used to analyze each quantitative gene expression phenotype from microarray analysis to genetic microsatellite and SNP markers. The linkage of an individual gene expression value as a continuous trait and a microsatellite marker was calculated with MERLIN variance component linkage analysis program. The potential regulatory regions were mapped as cis-regulatory if within 5 Mb of the gene, and trans-regulatory, if >5 Mb from the gene (Morley et al. 2004). Two nominal differential expressed genes within the haplotype (set 1: *AGA* aspartylglucosaminidase, *GALNT7* UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase *7*) were tested for linkage to markers on chromosome 4. Potential *trans*-regulators of 200 of the most significant differentially expressed genes with high coefficient of variation and located outside the schizophrenia haplotype (set 2: 200 genes) were examined for linkage to chromosome 4q genetic markers. To investigate *trans*-regulation further, all the remaining differentially expressed probesets throughout the genome (set 3: 1,327 genes) were analyzed for linkage to chromosome 4 markers. There were 153 SNPs from 150–191 Mb on chromosome 4 and 29 microsatellite markers spanning the entire chromosome that were used to detect linkage of gene expression to the 4q33–35.1 haplotype. Although these methods have application to genome-wide studies, this report focuses on linkage studies conducted mainly on the chromosome 4 results. A separate report of genome-wide linkage to genome-wide gene expression is in

Table 3 Demographics of postmortem Stanley microarray dorsolateral prefrontal cortex samples

After processing all microarrays, the highest quality microarrays ($n = 88$) were used to conduct analysis of covariance using group and gender as main factors, with age and pH used as covariates. The results for all genes in the chr 4q33–35.1 region are shown in Table 4

Demographics	Group					
	Bipolar		Control		Schizophrenia	
	Mean	SD	Mean	SD	Mean	SD
Age	45.4	10.7	43.3	7.4	42.6	8.4
PMI	37.8	18.4	29.6	12.5	29.9	14.8
pH	6.45	0.25	6.59	0.27	6.46	0.24
Race						
White	32		36		35	
Black	1		0		0	
Native american	1		0		0	
Hispanic	0		0		1	
Gender						
Female	17		11		9	
Male	17		25		27	
Total subjects	34		36		36	

preparation to analyze the results of the more extensive calculations involving $\sim 5 \times 10^8$ comparisons of genetic markers to gene expression.

Direct DNA sequencing of candidate genes

Candidate genes within the linkage peak were selected for mutational analysis. Individual genes (*AGA, FLJ22649, HAND2*) were scanned for known and unknown SNPs. Affected individuals with the chromosome 4q haplotype and unaffected individuals without the haplotype were used for sequencing. DNA was amplified by PCR followed by fluorescent sequencing. Briefly, the primers were designed using Oligo 6.0 (Molecular Biology Insights) and purchased from Applied Biosystems. DNA fragments were resolved via electrophoresis on a 1% agarose gel, and bands were visualized (ChemiDoc digital imaging system, BioRad, CA, USA) and extracted from the gel with the Qiagen Gel Extraction Kit (Qiagen Inc., CA, USA). Each fragment was sequenced using fluorescently labeled dye terminator sequencing chemistry (Big Dye

Table 4 Comparison of lymphocyte and DLPFC (Stanley Foundation) gene expression for 4q33–35.1

Lymphocyte U133A chip				DLPFC CodeLink 20 K				
UGCluster	*P* (schizoprenia vs. control) ANCOVA	Fold change (schizoprenia vs. control)	Symbol	Cytoband	*P* (schizoprenia vs. control)	Fold change (schizoprenia vs. Control)	*P* value (bipolar vs. control)	Fold change (bipolar vs. control)
Hs.126838	0.840	0.989	ADAM29	4q34	NA	NA	NA	NA
Hs.207776	0.100	0.851	**AGA**	4q32–q33	**0.046**	**1.263**	0.274	1.135
Hs.127407	0.170	1.394	GALNT7	4q31.1	0.890	0.986	0.744	1.033
Hs.413099	0.879	1.002	GLRA3	4q33–q34	0.991	0.999	0.766	1.031
Hs.75819	0.798	0.958	GPM6A	4q34	0.493	0.844	0.739	0.921
Hs.75819	0.798	0.958	GPM6A	4q34	0.546	0.947	0.637	0.959
Hs.75819	0.798	0.958	GPM6A	4q34	0.889	1.008	0.068	0.895
Hs.388245	0.763	1.036	HAND2	4q33	0.205	0.898	0.176	0.891
Hs.388245	0.763	1.036	HAND2	4q33	0.437	0.857	0.594	0.902
Hs.434953	0.771	0.927	**HMGB2**	4q31	**0.005**	**1.346**	**0.005**	**1.344**
Hs.77348	0.347	0.979	HPGD	4q34–q35	0.066	1.619	0.166	1.444
Hs.77348	0.347	0.979	HPGD	4q34–q35	0.976	0.997	0.408	0.917
NA	NA	NA	MORF4	4q34.1	0.823	1.024	0.007	0.743
Hs.413835	0.700	1.076	SAP30	4q34.1	0.652	1.034	0.702	0.972
Hs.7122	0.165	0.950	**SCRG1**	4q31–q32	**0.021**	**1.260**	0.571	1.057
Hs.42194	0.823	0.974	SPCS3	4q34.2	NA	NA	NA	NA
Hs.435215	0.074	1.120	VEGFC	4q34.1–q34.3	0.790	1.064	0.306	1.268

Both datasets were analyzed with an analysis of covariance to adjust for age. There were multiple probes for the same gene on Codelink, so GPM6A, HAND2, and HPGD results are shown. There were three genes differentially expressed in DLPFC in schizophrenia are shown in bold: *AGA* aspartylglucosaminidase, *HMGB2* high-mobility group box 2, and *SCRG1* Scrapie responsive protein 1. AGA was differentially expressed in DLPFC and lymphocyte in schizophrenia, although in lymphocyte the expression was not significant after ANCOVA

NA gene not on array platform

Terminator Cycle Sequencing v.3.1, Applied Biosystems) using capillary gel electrophoresis (ABI Prism 3100).

Oligonucleotide expression microarray

Total RNA from human lymphoblastoid cells was reverse-transcribed to cDNA using a T7-$(dT)_{24}$ (HPLC purified) primer (Superscript cDNA Synthesis Kit, Invitrogen). The cDNA was cleaned (Qiagen Gene Chip Sample Cleanup Module) and synthesized into cRNA (MegaScript T7, Ambion, TX, USA), incorporating biotinylated-UTP and biotinylated-CTP (PE Lifesciences, MA, USA). Biotinylated cRNA was cleaned (Qiagen Gene Chip Sample Cleanup Module), quality-checked (Bioanalyzer 2100, Agilent), fragmented, and hybridized to the Affymetrix Human Genome U133A array. Arrays were washed, stained, and scanned on the Affymetrix Fluidics Station and Gene Scanner. The gene expression traits were derived from the U133A chips and analyzed with robust multiarray condensation algorithm (RMA; Irizarry et al. 2003). Differential gene expression (gene expression trait for the purpose of this analysis) was defined as a gene that displayed a significant two-tailed t test ($P < 0.05$) in schizophrenia compared to unaffected family members.

Q-PCR

Primers were designed within a 3′ exon using the Primer Express program (Applied Biosystems). A nucleotide BLAST (NCBI) was performed to check the specificity of the primers. cDNA for quantitative real-time PCR was synthesized from the same human total RNA extracted from lymphocytes and used in microarray analysis. A 1:100 dilution of cDNA (5 µl) was added to each Q-PCR reaction containing SYBR Green PCR Master Mix (Applied Biosystems), and amplification was carried out on the 7000 Sequence Detection System (Applied Biosystems). A standard curve used to quantitate the products was generated from dilutions of known concentrations of genomic DNA. All samples and standards were run in triplicate. The average values were used for

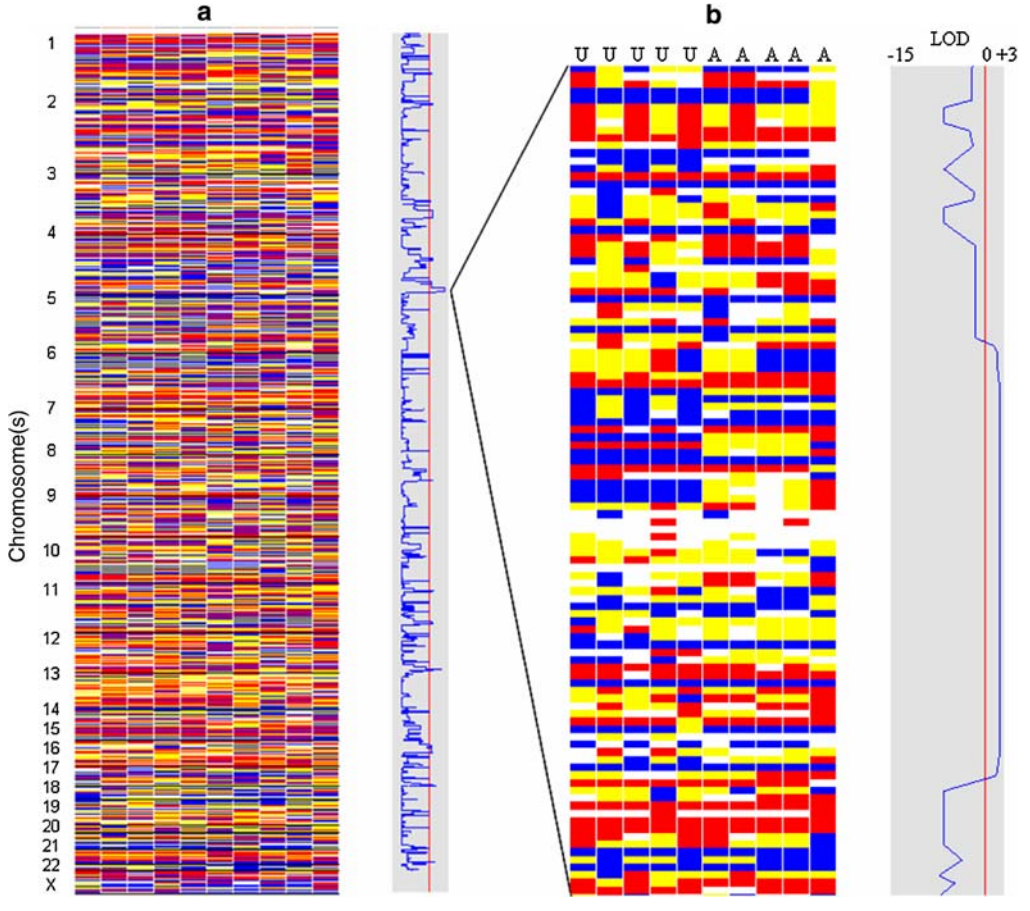

Fig. 2 Linkage analysis of schizophrenia for Affymetrix 10 K SNP markers is shown for all chromosomes (**a**). The maximum LOD peak was 2.39 ($\theta = 0$) at 4q33.1–34.3 assuming a dominant model. Genotypes within the highest LOD peak are enlarged (*U* unaffected, *A* affected) (**b**). The *shaded area* represents an LOD interval of (−15 min to 3 max), and the *red line* threshold is LOD = 0. The *color codes* for each subject shown depict genotype: *red box* = A, *blue box* = B, *yellow box* = AB, *white box* = no call

t tests to compare control and schizophrenia copy number before and after correction for a housekeeping gene (*SLC9A1*). The overall results did not change after normalization.

Results

Genome-wide scan with microsatellite and SNP markers

First, a low-resolution genome-wide linkage analysis was carried out with ~329 RFLP and microsatellite markers. The multiplex pedigree (Table 1, Fig. 1) showed a suggestive linkage peak in the 4q region (D4S1530, LOD 2.17, $\theta = 0$) using a dominant model.

Haplotype analyses using flanking microsatellite markers delineated a 14 Mb region that cosegregated with all schizophrenic cases. In order to determine whether other linkage signals may have been missed with the initial low-resolution scan, we carried out a higher-resolution SNP scan by microarray similar to another linkage study that also used both microsatellite markers and SNP microarray (Middleton et al. 2004). A linkage peak from 171.2–185.1 Mb (NCBI physical map) was mapped with a multipoint maximum LOD of 2.39 with the Affymetrix 10 K SNP markers (Fig. 2). The SNP linkage results showed a haplotype that segregated with the disease in this pedigree (Table 2). The linkage analysis was conducted with both MERLIN and dCHIP Linkage, and the results agreed for the region on 4q that showed suggestive linkage. MERLIN linkage using a dominant parametric model showed a maximum LOD = 2.56. To correct the SNP linkage data for linkage disequilibrium (LD), we reanalyzed the SNP linkage markers with several cluster options using MERLIN; however, there was a recombination in the region that prevented correction for LD in this pedigree.

Overall, the region on chromosome 4q implicated by both microsatellite 171.2–185.1 Mb and SNP linkage 171.8–184.6 analyses was consistent. Recombination inferred by both microsatellite marker and SNP methods defined the haplotype interval. The SNP haplotype that segregated with the affected in the 4q33–35.1 region is shown (Fig. 2).

Differentially expressed genes lymphocytes

In the latest build of Unigene (March 2006) there are approximately 125 Unigene clusters in the 4q33–35.1 region. The Affymetrix U133A GeneChip chip definition file incorporated 12 known genes within the region. The expression results were condensed using RMA and evaluated for statistical significance with a simple un-moderated *t* test. There were 1,340 genes significantly different ($P < 0.05$) between the affected and unaffected groups. Two genes were differentially expressed, *AGA* and *GALNT7*,and were both within the chromosome 4q33–35.1 region.

Table 5 Two genes within the haplotype that showed differential expression were analyzed for linkage to genetic markers on a genome-wide scan. *GALNT7* showed linkage to markers outside the chromosome 4q33–35.1 region (LOD = 3.15), and *AGA* showed suggestive linkage to markers within the haplotype region (LOD = 2.37). Bold LOD scores >2.3 are shown for *GALNT7* and *AGA*

Gene symbol	Gene physical position (Mb)	Genetic marker (microsatellite or SNP)	Marker physical position	LOD	*P* value	*Cis*-regulatory position
GALNT7	174,785,845	rs538307	4:83,182,269	1.51	0.004	91,603,576
GALNT7	174,785,845	rs1103021	4:117,447,717	**2.95**	0.00011	57,338,128
GALNT7	174,785,845	rs724749	4:126,184,175	**2.75**	0.0002	48,601,670
GALNT7	174,785,845	rs951530	4:126,242,981	**3.15**	0.00007	48,542,864
GALNT7	174,785,845	rs724156	4:130,903,653	**2.9**	0.00013	43,882,192
GALNT7	174,785,845	rs1876054	4:133,497,030	**2.87**	0.00014	41,288,815
GALNT7	174,785,845	rs1378149	4:187,549,919	1.56	0.004	12,764,074
GALNT7	174,785,845	rs1280100	4:188,234,023	1.52	0.004	13,448,178
GALNT7	174,785,845	rs952178	4:189,221,787	1.55	0.004	14,435,942
GALNT7	174,785,845	D16S83	16:1,054,534	2.3	0.0004	–
GALNT7	174,785,845	UT581	16:3,310,331	2.01	0.001	–
AGA	179,047,814	rs1318822	4:170,460,379	2.03	0.0011	8,587,435
AGA	179,047,814	rs1403225	**4:171,867,785**	1.85	0.002	7,180,029
AGA	179,047,814	dbSNP_12[a]	**4:173,507,703**	1.85	0.002	5,540,111
AGA	179,047,814	dbSNP_13[b]	**4:173,507,717**	1.9	0.002	5,540,097
AGA	179,047,814	rs1812424	**4:173,851,484**	1.85	0.002	5,196,330
AGA	179,047,814	D4S2431	**4:175,095,302**	1.52	0.004	3,952,512
AGA	179,047,814	UT1950	**4:181,860,360**	**2.37**	0.0005	2,812,546

GALNT7 UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 7 (GalNAc-T7); Affymetrix probeset-U133A 218313_s_at

AGA aspartylglucosaminidase; Affymetrix probeset-U133A 204332_s_at

[a]dbSNP_12 corresponds to Affymetrix SNP probe 1508870

[b]dbSNP_13 corresponds to Affymetrix SNP probe 1509404

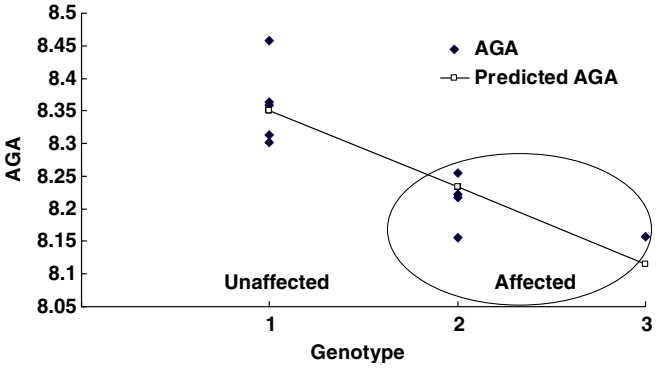

Fig. 3 The AGA microarray gene expression is plotted by genotype for SNP rs723819 (*1* homozygous for the major allele; *2* heterozygous; *3* homozygous for the minor allele). There was no overlap in genotype or microarray expression

After ANCOVA adjustment of expression means for RNA quality and age, the number of significantly differentially expressed genes was reduced to 451. We are using a custom chip definition file, so that only 11,282 Unigene clusters are represented on the U133A chips after a stringent BLAST of each Affymetrix designed probe (Dai et al. 2005). The significant 451 genes did not survive correction for multiple testing. Three genes in the chromosome 4q haplotype showed a trend for differential expression (*AGA*; *GALNT7*; vascular endothelial growth factor C *VEGFC*; $P \leq 0.1$) after ANCOVA adjustment for age and microarray chip quality (3′/5′ GAPDH ratio) as covariates.

Differentially expressed genes postmortem dorsolateral prefrontal cortex

A microarray study of DLPFC from the Stanley Foundation (Table 3) used 34 subjects with bipolar disorder, 36 subjects with schizophrenia, and 36 controls was run on the Codelink 20 K platform (GE Health). The 88 highest quality microarray samples were used for an analysis of covariance using group and gender as main factors, and age and pH as continuous covariates. The expression of all genes on the microarray platforms within the 4q33–35.1 region for both brain and lymphocytes is shown (Table 4). There were three genes differentially expressed in DLPFC in schizophrenia in the 4q33–35.1 region: AGA, HMGB2 (high-mobility group box 2), and SCRG1 (Scrapie responsive protein 1). AGA was differentially expressed in DLPFC and lymphocyte in schizophrenia, although in lymphocyte the expression was not significant after ANCOVA.

Linkage analysis of microarray gene expression

The quantitative gene expression values were used for calculation of genome-wide linkage with a variance components analysis in MERLIN (Table 5).

Genome-wide linkage of the two differentially expressed genes in the haplotype (set 1) showed a maximum LOD score of 2.37 for *AGA* to a single marker (UT1950) in the haplotype (Table 5). Linkage for *AGA* was computed with SNP markers and *AGA* showed evidence of *cis*-regulatory influence. Figure 3 demonstrates the association between *AGA* expression and SNP rs723819 genotypes (177.6 Mb) in the unaffected (genotype 1) compared to the affected (genotypes 2 and 3). However, *AGA* also showed evidence of *trans*-regulation (Table 5) on chromosome 4 with an LOD of 2.03 to SNP rs1318822 located 8.9 Mb upstream of the gene.

The second differentially expressed gene, *GALNT7*, displayed evidence of suggestive linkage to an area near the 4q33–35.1 region, and evidence of linkage (LOD = 3.15) to a regulatory control locus at SNP marker rs951530 (chromosome 4:126,141,720). *GALNT7* also showed suggestive linkage to chromosome 16 (Table 5). Thus, *AGA* was the only gene within the schizophrenia haplotype region that showed linkage (LOD = 2.37) to a potential *cis*-regulator.

To discover additional gene expression traits that might be coregulated within the linkage region, a set of differentially expressed genes was used for a similar linkage analysis. The top 200 differentially expressed genes (set 2) with the highest coefficients of variation

Table 6 Two hundred differentially expressed genes were scanned for linkage to markers within the haplotype. *ADH1B* showed evidence of a strong *trans*-regulatory loci at 162 Mb with a maximum LOD = 3.08. *ADH1B* also showed evidence of suggestive linkage within the 4q33–35.1 region shown in bold in physical position

Gene symbol	Chromosome 4 NCBI (Mb)	Genetic marker (microsatellite or SNP)	Physical position	LOD	*P* value	*Cis*-regulatory position
ADH1B	100,537,000	FGA	155,800,000	**2.7**	0.0002	55,263,000
ADH1B	100,537,000	rs1343937	156,388,728	**2.84**	0.0002	55,851,728
ADH1B	100,537,000	rs719880	162,724,739	**3.08**	0.00008	62,187,739
ADH1B	100,537,000	rs355191	167,251,943	2.07	0.001	66,714,943
ADH1B	100,537,000	rs191644	167,252,139	2.07	0.001	66,715,139
ADH1B	100,537,000	rs2319145	168,956,566	2.11	0.0009	68,419,566
ADH1B	100,537,000	rs2333244	**177,246,433**	2.27	0.0006	76,709,433
ADH1B	100,537,000	rs1368871	**180,251,308**	2.26	0.0006	79,714,308
ADH1B	100,537,000	rs1368870	**180,251,647**	2.26	0.0006	79,714,647

ADH1B alcohol dehydrogenase 1B (class I), beta polypeptide; Affymetrix probeset-U133A 209614_at

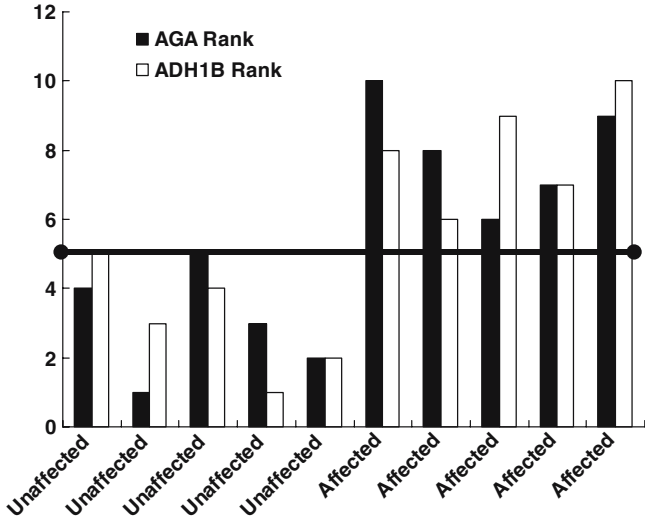

Fig. 4 The expression levels of AGA and ADH1B genes did not overlap between the affected and the unaffected, and showed linkage to chromosome 4 markers in the schizophrenia locus. AGA is located within the putative schizophrenia locus on 4q and showed evidence of *cis*-regulation

across all samples were selected (Cheung et al. 2003; Cheung and Spielman 2002). This second subset was tested for linkage to chromosome 4q33–35.1 markers. Linkage results for the top 200 dysregulated genes to markers within the chromosome 4q haplotype showed one gene, alcohol dehydrogenase IB (*ADH1B*), with an LOD of 2.27 (Table 6). However, another *trans*-regulatory locus for *ADH1B* showed an LOD score of 3.08 (rs719880 located at chr4: 162,504,718). We cannot presently resolve whether these regulatory loci on chromosome 4 at 162 and 177 Mb represent the same potential control locus or these are two different regulatory loci. There was no overlap in expression between affected and unaffected individuals for gene expression of *ADH1B* (Fig. 4).

In the third set, the remaining 1,327 differentially expressed probesets from the RMA *t* test genome wide were analyzed and produced three gene probesets showing suggestive linkage (LOD > 2.3) to SNPs on chromosome 4 within the haplotype. The three genes that showed linkage to SNPs within the chromosome 4 haplotype are: *ZNF291*, *CSNK1A1*, and *MSCP* (Table 7). However these genes did not show differential expression following ANCOVA adjustment. A complete analysis of all gene expression linkages to SNP genotype markers would require $\sim 5.2 \times 10^8$ calculations, the main focus for this paper was in the schizophrenia haplotype region.

Q-PCR validation of differentially expressed genes

Genes with nominal differential expression between unaffected and affected family members were tested by real-time Q-PCR with SybrGreen. The Q-PCR fold changes for schizophrenia compared to controls were larger than Q-PCR fold-changes that were observed with microarray (Table 8). For example, *ADH1B* was validated by Q-PCR (Table 8) and showed a 9.5-fold decrease in the affected versus unaffected family members. However, *AGA* and *GALNT7* displaying robust fold changes consistent with microarray did not reach statistical significance. The overall validation of candidate genes tested in this study was 7 out of 14 genes tested indicating about 50% validation rate of microarray data.

Mutation screening

Direct sequencing of all coding regions, exon–intron junctions and possible regulatory regions for *FLJ22649*, *HAND2*, and *AGA* was performed on the five affected and five unaffected family members. There was no potential disease predisposing mutation found in these genes that segregated with schizophrenia in this pedigree. Nine previously published exonic SNPs were genotyped in the same sample set. There was no clear allelic segregation detected based upon illness.

Discussion

This study used a combined approach of linkage to identify a schizophrenia susceptibility region and gene expression to identify candidate genes within the linkage region. The genome-wide scan with microsatellite markers and SNPs suggested linkage of schizophrenia to chromosome 4q33–35.1 in a high-density multiplex pedigree. The pedigree was followed up with microarray

Table 7 The remaining differential expressed probesets in schizophrenia compared to the unaffected were also analyzed for linkage to the chromosome 4 haplotype. There were no genes that showed above an LOD score > 2.5

Gene	Cytogenetic band	Affymetrix probeset	dbSNP ID	Kong and Cox LOD[a]	*P* value	SNP location
ZNF291	15q24	215848_at	DBSNP_13[b]	2.32	0.0005	172.8
CSNK1A1	5q32	208867_s_at	RS2333244	2.42	0.0004	176.4
MSCP	8p21	218978_s_at	RS1902018	2.48	0.0004	178.5
MSCP	8p21	218978_s_at	RS1454214	2.46	0.0004	178.5

[a]Calculated in MERLIN linkage (Abecasis et al. 2002)
[b]D4S1308 /// D4S2328. dbSNP_13 corresponds to Affymetrix SNP probe 1509404

Table 8 Q-PCR of genes that showed differential microarray expression

Gene symbol	Microarray fold change	Q-PCR fold change	Microarray (*P* value *t* test)	Q-PCR (*P* value *t* test)	Cytogenetic band	Gene name
ADAM9	**1.45**	**1.70**	**0.024**	**0.04**	8p11.22	A disintegrin and metalloproteinase domain 9 (meltrin gamma)
ADH1B	**0.89**	**0.10**	**0.023**	**0.02**	4q21–q23	Alcohol dehydrogenase IB (class I), beta polypeptide
AGA	0.88	0.77	0.022	0.42		
CTLA4	**0.82**	**0.14**	**0.036**	**0.04**	2q33	Cytotoxic T-lymphocyte-associated protein 4
FLJ20637	0.81	0.68	0.014	0.08	4q22.1	Hect domain and RLD 6
G3BP2	1.28	2.04	0.045	0.07	4q21.21	Ras-GTPase activating protein SH3 domain-binding protein 2
GALNT7	1.34	2.81	0.041	0.46	4q34.1	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 7 (GalNAc-T7)
HCK	**2.58**	**4.71**	**0.030**	**0.02**	20q11–q12	Hemopoietic cell kinase
HERC3	0.89	0.90	0.023	0.34	4q21	Hect domain and RLD 3
IGJ	0.45	0.63	0.038	0.21	4q21	Immunoglobulin J polypeptide, linker protein for immunoglobulin α and μ polypeptides
NR2F2	**1.74**	**4.15**	**0.047**	**0.02**	15q26.2	Nuclear receptor subfamily 2, group F, member 2
NS3TP2	1.27	1.75	0.030	0.08	5q23.3	HCV NS3-transactivated protein 2
SMARCA2	**1.75**	**2.27**	**0.028**	**0.03**	9p22.3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
SNX2	1.43	0.95	0.042	0.45	5q23	Sorting nexin 2
TMOD3	1.21	1.39	0.031	0.12	15q21.1–q21.2	Tropomodulin 3 (ubiquitous)
WSB2	**1.49**	**2.18**	**0.003**	**0.02**	12q24.23	WD repeat and SOCS box containing protein 2

ADH1B showed a significant difference between the affected and unaffected by Q-PCR. *ADH1B* was confirmed by Q-PCR and showed an LOD score of 3.0 for regulatory control locus just outside of the haplotype and a secondary locus inside of the haplotype. Of 14 candidate genes tested 7 were confirmed (shown in bold) indicating a 50% validation rate of the microarray data, an additional three genes showed a trend at *P* value <0.1 for validation

screening of gene expression. We were not able to demonstrate a significant linkage between the putative haplotype for disease and for gene expression. This may be due to a suggestive but nonsignificant LOD score generated for schizophrenia in this single pedigree. Thus, we emphasize that these results indicate the methods of using gene expression and linkage with a disease to attempt to pinpoint regulatory and possibly causative loci.

We found that *ADH1B* differential gene expression is validated by Q-PCR, and showed a significant LOD score to a regulatory locus. After considering all 1,327

Table 9 Summary of genetic studies in schizophrenia reporting findings on chromosome 4q

Marker	NCBI (Mb)	LOD (Zmax)	Reference
D4S2361	85	1.3	Paunio et al. (2001)
D4S35	91–96	>1.0	Coon et al. (1994)
D4S2623	111	1.36	Levinson et al. (1998)
D4S2623	111	1.43	Mowry et al. (2000)
4q22–31	111	3.0	Gurling et al. (2001)
D4S2917	109	2.64	Reported in Kennedy and Macciardi (1998)
D4S2394	130	1.04	Paunio et al. (2001)
D4S2394	130	0.76, 1.40, 1.19	Ekelund et al. (2000)
D4S2639	185	0.62, 0.83, 1.13	Stages I, II, and III
D4S1564	118.96	(1.54)	Kaufmann et al. (1998)
D4S2395	134.97	(1.68)	
D4S1644	151.48	(1.54)	
D4S424	142	(0.34)	Hovatta et al. (1999)
D4S1586	158	(2.74)	
D4S3046	163	(0.29)	
D4S3250–D4S3335	124–185	1.84	Straub et al. (2002)
D4S1625	144	1.67	
D4S2417	147	1.50	
D4S2631	180.8	1.2	
D4S3335	156.3	1.2	
	185	0.96	
D4S1535	185	2.3	Fallin et al. (2003)
D4S415	179		
D4S1539	176		

nominally significant gene expression differences for linkage to chromosome 4, both *ADH1B* and *GALNT7* showed significant LOD scores for linkage to chromosome 4 (LOD > 3.0). Interestingly, both genes showed suggestive linkage of a smaller magnitude to the haplotype region (LOD > 2.0). We focused our search on chromosome 4q haplotype, as an exhaustive analysis of all gene expression and SNP markers would require $\sim 5 \times 10^8$ statistical tests. However, with additional subjects it would be worthwhile to pursue a genome-wide scan of gene expression regulatory loci.

Gene expression in peripheral white blood cells has been tested in schizophrenia, bipolar, and controls (Tsuang et al. 2005; Middleton et al. 2005). Linkage regions in schizophrenia and bipolar disorder have been further scanned for changes in gene expression of peripheral white blood cells (Middleton et al. 2005). We have also found that three genes within the 4q33–35.1 region are differentially expressed in DLPFC (*AGA*, *SCRG1*, and *HMGB2*). Thus, another potential use for this type of investigation is for determining whether the lymphocyte expression differences serve as potential biomarkers, especially if the gene is also expressed in the brain.

Several genetic studies of schizophrenia have reported positive linkage findings on chromosome 4q (Hovatta et al. 1999; Paunio et al. 2001; Levinson et al. 1998; Mowry et al. 2000; Kennedy and Macciardi 1998; Ekelund et al. 2000; Kaufmann et al. 1998; Table 9). Evidence of suggestive linkage was reported to the same 4q region as the present study (Straub et al. 2002; Fallin et al. 2003). However, in a meta-analysis of schizophrenia linkage studies, chromosome 4q was not significant (Lewis et al. 2003); thus, the 4q region may only account for a small fraction of disease liability.

We have used Q-PCR to validate the microarray data. However, we were unable to validate two candidate genes in the 4q33–35.1 region. In reanalysis of the data using unequal variance, the *t* test for both genes became nonsignificant. Thus, the Q-PCR failure was due to high variation in samples. However, we were able to validate 7 out of 14 genes tested by Q-PCR.

ADH1B might be a candidate gene based upon gene expression differences in schizophrenia and linkage to regulatory loci within the schizophrenia haplotype and outside of the haplotype. *ADH1B* metabolizes substrates in pathways involving serotonin, norepinephrine, dopamine, and alcohol (Consalvi et al. 1986; Helander et al. 1994; Svensson et al. 1999; Matsuo and Yokoyama 1989; Matsuo et al. 1989) and thus is relevant to psychiatric disorders. Although we did not find a predisposing mutation in any exons of *AGA* for schizophrenia, there are known mutations of *AGA* that cause the lysosomal storage disease, Aspartylglucosaminuria (OMIM# 208400). Aspartylglucosaminuria is characterized by predominant cognitive deterioration, mental retardation, and emotional lability.

Potential regions for control of gene expression were mapped to *cis*- and *trans*-regulatory sites. The results of the study are consistent with prior reports demonstrating that gene expression traits in lymphocytes are heritable in multiplex pedigrees (Morley et al. 2004; Monks et al. 2004; Schadt et al. 2003a, 2003b). These results suggest that studying gene expression traits in combination with linkage studies helps identify regulatory regions for candidate genes. A high LOD score between a quantitative trait (gene expression) and a genetic marker suggests that possible regulatory elements within the linkage region may regulate the quantitative trait (gene expression) (Schadt et al. 2003a). Schadt et al. (2003a, 2003b) reported that 423 genes were linked to a chromosome 2 locus of interest, which was a quantitative trait of subcutaneous fat deposition. Notably, only four genes were within 2 cM of the peak where gene expression traits showed linkage. "Most of the genes linked to the chromosome 2 locus do not physically reside on chromosome 2, and so, are at least partially regulated by one or more loci in the chromosome 2 hotspot region" (Schadt et al. 2003a). This reasoning can apply to the present results, as most genes with differential expression showed regulatory loci in a *trans*- location, while some genes also showed evidence of being partially regulated by two distinct loci.

The differentially expressed gene, such as *ADH1B*, located outside of the haplotype but linked to the haplotype may not confer risk to illness, but may modify gene expression. This modification in gene expression could confer a variation in symptoms, onset, or subtype in the schizophrenia syndrome. Consistent with the Schadt et al. (2003a, 2003b) study, we find differentially expressed candidate genes on other chromosomes with suggestive linkage to the haplotype (Table 5). Both *cis*- and *trans*- acting factors regulate genes, perhaps in combination in a complex disorder, as suggested by our results and others (Morley et al. 2004). Improvements in the analytical methods are needed to integrate differential gene expression as a phenotype and linkage to genomic markers (Schadt et al. 2003a, 2003b; Kraft et al. 2003; Sham et al. 2002; Horvath and Baur 2000; Middleton et al. 2005). However, taken together, linkage analysis of the inheritability of gene expression traits will be useful for mapping regulators of gene expression.

Acknowledgements This work was supported by the William Lion Penzner Foundation (UCI Department of Psychiatry and Human Behavior) and the NIMH RMH074307A award (MPV). Postmortem brain tissue was donated by The Stanley Medical Research Institute's brain collection courtesy of Dr. Michael B. Knable, Dr. E. Fuller Torrey, Dr. Maree J. Webster, Dr. Serge Weis, and Dr. Robert H. Yolken.

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