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Microarray screening of lymphocyte gene expression differences in a multiplex schizophrenia pedigree

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Abstract

In order to help prioritize the selection of candidate genes and to study possible trait and not state related changes in gene expression, we compared lymphocytic gene expression patterns of five individual family members with schizophrenia and nine unaffected individuals from a large multiplex high density pedigree. We screened gene expression by microarray consisting of 1128 brain focused genes. Three criteria for selection of microarray gene differences between schizophrenia and unaffected family members were employed: a significant *t*-test, expression in a majority of subjects, and fold change magnitude. Gene expression levels were significantly different for nine genes between individuals with schizophrenia compared to unaffected controls, and two genes were validated by real-time PCR. The expression of the neuropeptide Y receptor Y1 gene (NPY1R localized at 4q31.3–q32) and the human guanine nucleotide-binding regulatory protein Go-alpha (GNAO1 localized at 16q13) was significantly decreased in individuals with schizophrenia compared to unaffected family controls by microarray and real-time PCR. The cytosolic malate dehydrogenase gene (MDH1 localized at 2p13.3) was also significantly increased by microarray analysis and showed a trend for increase by real-time PCR. The significant genes are discussed in terms of proximity to linkage regions, prior association studies of schizophrenia, and other reports of microarray screening of schizophrenia tissue. Evidence from these studies taken together with the present study suggests critical pathways in schizophrenia may be studied in peripheral tissue as part of the strategy in functional genomic convergence. This preliminary study needs to be repeated by screening a larger set of genes in additional families with schizophrenia. The present study offers support for examination of gene expression patterns using lymphocytic RNA for complex neuropsychiatric disorders from large cohorts of patients.

Keywords: Transformed lymphocytes; Microarray; Gene expression; GNAO1; NPY1R; MDH1; Real-time PCR

1. Introduction

Changes in gene expression and protein expression levels have been observed in the postmortem brain tissue of individuals with schizophrenia. Until recently, most postmortem studies only examined a few or several genes in one experiment. Recent advances in microarray technology make it possible to examine expression of up to 10,000 genes in one brain sample (Freed and Vawter, 2001). Differences in gene expression in the prefrontal cortex of unrelated patients with schizophrenia have been found by

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microarray and some of the observed changes may be related to the pathophysiology of schizophrenia (Hakak et al., 2001; Mirnics et al., 2000; Mimmack et al., 2002). Although the platforms utilized in these recent microarray studies varied, some genes have been consistently implicated, in particular, presynaptic genes, metabolic and ubiquitin pathway genes (Mirnics et al., 2000; Vawter et al., 2001; Middleton et al., 2002; Hemby et al., 2002).

While there is some agreement across studies in the genes differentially expressed in the prefrontal cortex samples of unrelated schizophrenics (Vawter et al., 2002), the lack of consistency in microarray results might also be related to etiological and genetic heterogeneity of the illness. Microarray studies are potentially important in examining common gene pathways that may be related to disease processes. The interpretations of microarray results must also account for state phenomenon, and variables associated with postmortem brain studies (agonal medications, hypoxia, pyrexia, agonal duration, postmortem interval, mRNA integrity, cellular heterogeneity of subcortical and cortical tissues, and anatomical inconsistency). Complex brain tissue will invariably contain the primary and secondary manifestations of the disorder while a single cell population with defined cellular phenotype might have some advantage over brain tissue in interpretation of the assessment of multiple genes.

There are no prior reports to our knowledge of possible genetically based alterations in lymphocytic gene expression within a high-density family study of schizophrenia. Lymphocytes express a number of brain related genes including the dopamine D3 and D5 receptors as well as other genes (Ilani et al., 2001; Kanzig, 2001; Kwak et al., 2001). It has been recently shown that lymphocytes express a large number of brain relevant genes using a cDNA microarray (M.P. Vawter, unpublished results). It is possible that brain related changes in gene expression might also occur in lymphoctyes.

In the present study, we used a cDNA microarray of 1128 human genes selected for relevance to brain function to examine the gene expression patterns of five patients with schizophrenia and nine nonaffected family members derived from a large multiplex pedigree. These preliminary results may be useful for the identification of biochemical markers in addition to candidate genes for further exploration of the biochemical pathways that may be involved in the pathophysiology of schizophrenia.

2. Methods

2.1. Ascertainment

This study was approved by the University of Utah and UCI Institutional Review Boards. Following informed consent, subjects were interviewed using a modified SADS-L; consensual RDC diagnoses were made by two psychiatrists without knowledge of the genotypes. The multiplex pedigree contains five individuals diagnosed with schizophrenia and is of Western/Northern European heritage. The pedigree was chosen as representative of a kindred with a high density of patients with schizophrenia using DSM-IV/RDC diagnostic criteria. The demographics of patients ($n = 5$) and unaffected control family members ($n = 9$) that were analyzed within the kindred are shown (Table 1).

Table 1
Demographic data for schizophrenia patients and unaffected subjects studied in multiplex schizophrenia pedigree

Group	Age (years)	Gender	Age of onset
Schizophrenia			
1	60	F	32
2	48	F	18
3	42	M	19
4	24	M	20
5	18	M	17
Unaffected control			
1*	16	M	
2	54	M	
3	48	M	
4	38	M	
5*	25	F	
6	51	F	
7	34	F	
8*	22	F	
9*	21	F	

The age in years represents the age of the subject when the lymphocytes were drawn and transformed. All subjects were initially used in the microarray data analysis. In a subgroup analysis of controls, those marked with '*' were excluded, since these younger controls have not passed the age of onset for schizophrenia and to ensure complete gender matching between groups.
M = male; F = female.

2.2. Microarray gene expression

Total RNA from patients with schizophrenia and unaffected control family members was obtained from transformed lymphocytes. The lymphocytes were transformed using the Epstein–Barr Virus and grown in a RPMI-1640 media supplemented with 15% fetal bovine serum (heat-inactivated), 2 mM L-glutamine and 25 mg of gentamicin and the cell lines were frozen. All 14 cell lines were thawed on the same date, and grown with the same batch of RPMI-1640 supplemented media under identical conditions (5% CO_2, 37 °C) to approximately the same cell density of 10^7 cells in a 25-cm^2 flask. The cells from the 25-cm^2 flask were split into two separate 75-cm^2 flasks and grown to a confluency of approximately 5×10^7 cells/flask. Freezer storage time and the time it took for the cells to grow to this confluency were not different between individuals with schizophrenia and unaffected family members (Table 2).

The transformed lymphoblastoid cell lines were harvested and the total RNA was extracted separately from each sample with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the supplier's protocol. Briefly, 1 ml of TRIzol is added to $5-10 \times 10^6$ cells, so 10 ml of TRIzol was used for each 50 ml confluent T75 flask of cells (pelleted). All final RNA pellets were resuspended in 100 μl of DEPC–water and RNA yields (resulting from the A260/280 readings) ranged from 90 to 390 μg of total RNA with the average yield being 250 μg.

Table 2
The number of days transformed lymphoblasts were grown and passaged during this study to confluency and the freezer storage time for each sample in the pedigree used for microarray analysis

Group	Incubator time (days)	Freezer storage time (months)
Schizophrenia		
1	23	146
2	~17	~158
3	21	156
4	23	158
5	22	158
Mean	22.2	155.2
Controls		
1	23	158
2	25	155
3	17	158
4	22	155
5	21	150
6	21	155
7	22	156
8	17	158
9	23	158
Mean	21.2	155.9

A sample of the total RNA was diluted to 1 μg/μl in DEPC–water. The resulting 28S and 18S ribosomal bands were visualized as well as any degradation using the Agilent BioAnalyser 2100 (Agilent, Palo Alto, CA). The 28S peak always represented a greater percentage of the total RNA than the 18S peak. The A_{260}/A_{280} ratio was >1.9 for all of the extracted RNA samples. As a final check of total RNA integrity and cDNA synthesis, real-time PCR for the 5′ beta-actin gene was also assessed.

2.3. RNA labeling and hybridization

Each sample was individually labeled and hybridized to individual microarrays. The entire labeling and hybridization procedures were repeated a second time. The procedure described for radioactive labeling of total RNA with [^{33}P]-dCTP was followed (Barrett et al., 2001). Briefly, total RNA (10 μg) is reverse-transcribed to cDNA with reverse transcriptase enzyme in the presence of [^{33}P]-dCTP. The [^{33}P]-dCTP-cDNA is purified through a spin column by size separation (BioSpin, Bio-Rad, California) and the heat-denatured probe ($\sim 5 \times 10^6$ cpm) is diluted in 4 ml of Microhyb solution (Research Genetics) and hybridized to the Neuroarray for 16–18 h at 50 °C with rotation. Two washes with 2× SSC at room temperature are carried out to remove unhybridized probe. The Neuroarray is placed under saran wrap and exposed to a low energy phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 5 days and scanned in a Phosphorimager 860 (Molecular Dynamics) at 50-μm resolution.

2.4. Neuroarray

The details of the development of the NIA-Neuroarray have been reported elsewhere (Barrett et al., 2001; Vawter et al., 2001). Briefly, a 15,000 human cDNA clone set of IMAGE Consortium clones http://image.llnl.gov/) available from Research Genetics (Huntsville, AL) was sorted for brain relevant genes and PubMed was consulted for relevant reports of

protein or gene expression screening of patients with neuropsychiatric disorders. This resulted in a list of genes (1128 clones) representing families such as transcription factors, synaptic, neuronal and glial proteins, cell adhesion molecules, kinases, phosphatases, proteases, oncogenes, and structural genes that were chosen for inclusion in the Neuroarray http://www.grc.nia.nih.gov/branches/rrb/dna/array.htm. DNA plasmids were PCR amplified with universal primers. The microarray printer (Genetic Microsystems 417, Woburn, MA) delivered ~5–10 nl of PCR reaction/spot in duplicate onto 42 dry Nytran+ Supercharge membranes (Schleicher & Schuell, Keene, NH) with individual spot diameter of ~300 μm (Fig. 1).

2.5. Statistical analysis

Arrayvision Software (Imaging Research, St. Catharines, Ontario, Canada) was used for semi-automated spot finding and to obtain average intensity of each cDNA target. The average intensity data was background-corrected with a two-pixel ring that surrounded the space between cDNA targets. The data was then calculated as a percent of total binding on each array. Each lymphocyte sample was labeled separately and run on individual microarrays. The experiment was duplicated by re-run of individual microarrays with newly labeled RNA. The data was analyzed by *t*-test analysis using the Cyber-T program (Baldi and Long, 2001; Long et al., 2001) available online at http://www.igb.uci.edu/servers/cybert/. To reduce the experiment wide false discovery rate, three criteria were established to identify differentially expressed genes between patients with schizophrenia and unaffected controls. All gene expression values had to be above background for at least 12/14 subjects so that the minimum number of subjects in each comparison had to be at least 12. The statistical criterion for a Student's *t*-test was $p < 0.05$ to be met for the mean difference between groups. The third criterion was that the magnitude of the fold change for the ratio (schizophrenia/control) must equal or exceed ± 1.4. Genes that met the three criteria (expression in 12/14 subjects, statistical threshold, and fold change magnitude) were considered differentially expressed between patients and controls.

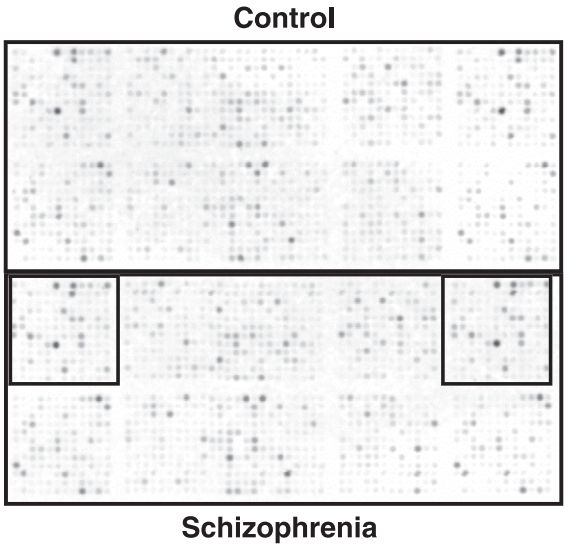

Fig. 1. Microarray hybridization of lymphocyte sample from an unaffected family member (upper panel) and individual with schizophrenia (lower panel). The cDNA microarrays were exposed to a phosphorimaging screen (see Methods for protocol). Inset box shows duplicate grids on upper left and upper right of the lower array. The spot diameter is approximately 300 μm.

A post hoc analysis of the subjects with schizophrenia ($n = 5$) and unaffected controls beyond the age of onset of schizophrenia ($n = 5$) and matched for gender was also conducted. Those controls that were excluded in the post hoc analysis are shown in Table 1 with an asterisk. The criteria for a significant difference in gene expression in this post hoc analysis using the control subgroup were the same (Student's *t*-test and fold change) as in the main group analysis with a complete control set of subjects. This analysis was conducted to eliminate gender and age as potential confounds in the data analysis and possible controls that might not yet manifest schizophrenia.

2.6. Real-time PCR

Total RNA from 14 lymphocyte samples (five patients with schizophrenia and nine family unaffected controls) were treated with DNase (Takara Bio, Otsu, Shiga, Japan) and subsequently purified with the RNeasy Mini Kit (Qiagen, Chatsworth CA) followed by ethanol precipitation. cDNA was synthesized by using Taqman® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) with an oligo $d(T)_{16}$ primer. Quantitative real-time PCR was performed using SYBR Green Dye (Applied Biosystems). Addi-

tional control for genomic DNA contamination of the sample was assessed by including a reverse transcription-negative control for each RNA sample. Each sample was further assessed for cDNA integrity by SYBR Green real-time PCR for the 3′ end of the beta-actin gene. PCR reactions were carried out in an Applied Biosystems 7000 sequence detection system (Applied Biosystems) according to the manufacturer's SYBR® Green PCR master mix protocol (25 μl reaction volume). Samples were quantified by comparison to a standard curve generated with known concentrations of PCR product from amplification of genomic DNA. The template concentrations for the gDNA standard curve ranged from 100 to 0.1 ng/μl in a 10-fold dilution series. For MDH, whose product spanned an intron–exon boundary, the standard curve was generated with known concentrations of PCR product from amplification in IMAGE Clone: 725188 (MDH 3′). The template concentrations for the MDH clone ranged from 3.5 to 3.5×10^{-7} ng/μl. cDNA added per PCR was normalized by comparing PCR amplification of the 3′ end of the beta-actin gene.

The primer sequences chosen for real-time PCR for MDH1 [malate dehydrogenase 1, NAD (soluble), AA403295], NPY1R [neuropeptide Y receptor Y1, R19478], GNAO1 [guanine nucleotide-binding protein (G protein), alpha activating activity polypeptide O,] and 3′ β-actin [actin, beta] are the following:

MDH1 forward primer 5′-CTGTGACCACGTCAGGGACAT-3′
MDH1 reverse primer 5′-TCTTGATTACAACAGGGAATGAGTAGAG-3′
NPY1R forward primer 5′-AAGCACAACCTGCAACATACTTTG-3′
NPY1R reverse primer 5′-AAATGATTTCAACCCCAGTCCTT-3′
GNAO1 forward primer 5′-TCTACAGAAATACACAGCCGTCAGTC-3′
GNAO1 reverse primer 5′-CCTGTCTAACCTACGACCCCAG-3′
β-actin forward primer 5-CCTTCGTGCCCCCCC-3′
β-actin reverse primer 5′-GGAGACCAAAAGCCTTCATACATC-3′.

Following all amplification reactions, an amplicon dissociation curve was run on the ABI 7000 to look for specific melting of the PCR product at the expected temperature. A curve with one specific fluorescent peak in the temperature range of 59 °C was evidence that the real-time PCR measurements of product were not being influenced by primer–dimer pairs. Nonspecific fluorescent peaks evident at lower temperatures required redesign of primer pairs for use in the assay.

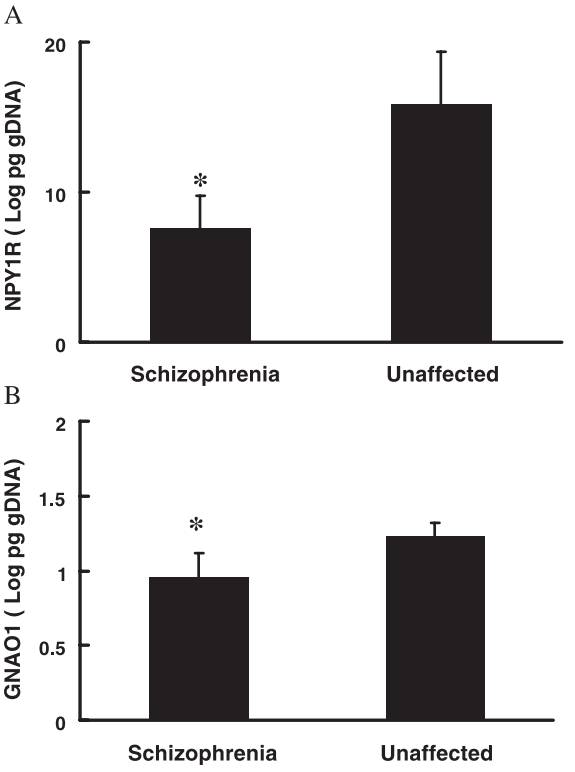

Fig. 2. Real-time PCR mean concentrations of NPY1R (A) and GNAO1 (B) in lymphocytes from patients with schizophrenia and unaffected controls. The gene expression levels shown are normalized to actin. The expression of both NPY1R and GNAO1 are significantly reduced in schizophrenia (*$p < 0.05$, one-tailed *t*-test), confirming the microarray results.

3. Results

3.1. Microarray gene expression

Fourteen of the affected and nonaffected family members from this high density family were screened for gene expression using individual microarrays for

each subject. There were nine genes that met the three criteria for differential expression in lymphocytes from patients with schizophrenia as compared to unaffected controls (Table 3). We found the neuropeptide Y receptor Y1 (NPY1R) was decreased in schizophrenia family members (-1.56 fold change; $p<0.05$). The NPY1R gene is located at 165 Mb (NCBI map) which is in a region of chromosome 4q32 near linkage demonstrated previously in a Finnish sample (Paunio et al., 2001). For comparison, the glutamate receptor, AMPA 2 (GRIA2) gene was found near this region but not significantly changed (fold change -1.27, $p<0.2$). The malate dehydrogenase 1, NAD soluble (MDH1) gene was increased in lymphocytes (fold change 1.46, $p<0.05$) and also shows decreased expression by microarray in DLPFC from patients with schizophrenia (Middleton et al., 2002; Vawter et al., 2003) and was chosen for further validation. The GNAO1 gene was decreased in schizophrenia (-1.57 fold change; $p<0.05$) and also has been found to be associated with schizophrenia shown in Table 3 (Tani et al., 2001) and was chosen for further validation.

There were an additional 21 genes that were significant by a *t*-test only ($p<0.05$) but did not exceed the threshold for fold change of ± 1.4 and above background expression in 12 of 14 subjects (Table 4). A frequency analysis of gene hits to Gene Ontology classifications and to the Kyoto Encyclopedia of Genes and Genomes pathways did not suggest overrepresentation of functional categories for these additional genes.

The post hoc control subgroup was formed to eliminate younger controls that may have not manifested schizophrenia and to balance gender, age, and number of subjects between groups. The resulting control group was composed of three males and two females, which balances the patient group (the eliminated controls are shown by asterisk in Table 1). In the post hoc analysis with the reduced number of subjects in the control group, all of the genes shown in Table 2, except NPY1R and PCDH2, continued to meet both the criteria of fold change magnitude and statistically significant *t*-test differences between patient and control groups. With reduced power in the post hoc comparison, seven of the nine genes remained significant, indicating that the gender and age of the controls were not responsible for the majority of the gene expression differences found between groups.

3.2. Real-time PCR gene expression

β-Actin gene expression was measured by real-time PCR in all lymphocyte samples. There was no difference between patients with schizophrenia and unaffected family members in levels of β-actin gene expression. β-Actin gene expression was used to normalize the expression measures of candidate genes. NPY1R gene expression normalized to β-actin was decreased in lymphocyte total RNA (Fig. 2; -1.52 fold change; one-tailed *t*-test, $p=0.042$) confirming the microarray results (-1.56 fold change; $p<0.05$). GNAO1 expression normalized to β-actin was also

Table 3
Differential gene expression for schizophrenia and unaffected controls by microarray analysis

Gene name	Gene symbol	Accession	Cytoband	Fold change
Decreased in patients with schizophrenia				
Amyloid beta (A4) precursor protein-binding, family A, member 3 (X11-like 2)	APBA3	W19429	19p13.3	−1.41
Neuropeptide Y receptor Y1	NPY1R	R19478	4q31.3–q32	−1.56
Human guanine nucleotide-binding regulatory protein (Go-alpha) gene	GNAO1	R43320	16q13	−1.57
Protocadherin gamma subfamily C, 5	PCDH2	R89615	5q31	−1.7
Increased in patients with schizophrenia				
Protein tyrosine phosphatase type IVA, member 2	PTP4A2	AA504327	1p35	1.40
Homeo box A13	HOXA13	AA147224	7p15–p14	1.41
Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	MPP3	W44685	17q12–q21	1.42
Malate dehydrogenase 1, NAD (cytosolic)	MDH1	AA403295	2p13.3	1.46

Significant gene expression difference between patients and unaffected controls for each gene is $p<0.05$. The fold change is greater than ± 1.4.

Table 4
Differential gene expression for schizophrenia and unaffected controls by microarray analysis

Gene name	Accession	Fold change	Gene symbol	Cytoband
Increased in patients with schizophrenia				
Fibroblast growth factor receptor 2	AA443093	1.3	FGFR2	10q26
Adaptor-related protein complex 3, sigma 1 subunit	AA460727	1.3	AP3S1	5q22
V-raf murine sarcoma 3611 viral oncogene homolog 1	H59757	1.3	ARAF1	Xp11.4–p11.2
Basic transcription factor 3	R83000	1.3	BTF3	5q13.1
Protein kinase C, iota	T57957	1.3	PRKCI	3q26.3
Potassium voltage-gated channel, Shab-related subfamily, member 1	AA069770	1.2	KCNB1	20q13.2
Protease, serine, 2 (trypsin 2)	AA284528	1.2	PRSS2	7q34
Capping protein (actin filament) muscle Z-line, beta	AA430524	1.2	CAPZB	1p36.1
Calcium/calmodulin-dependent protein kinase I	H29415	1.2	CAMK1	3p25.3
Crystallin, zeta (quinone reductase)	R13434	1.2	CRYZ	1p31–p22
Carbohydrate (keratan sulfate Gal6) sulfotransferase 1	R15740	1.2	CHST1	11p11.2–p11.1
V-ral simian leukemia viral oncogene homolog B (ras related; GTP-binding protein)	W39343	1.2	RALB	2cen-q13
Decreased in patients with schizophrenia				
Paxillin	AA430573	−1.2	PXN	12q24
Integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170), macrophage antigen alpha polypeptide)	AA436187	−1.2	ITGAM	16p11.2
Ephrin-B2	AA461424	−1.2	EFNB2	13q33
Butyrylcholinesterase	AA885311	−1.2	BCHE	3q26.1–q26.2
Phospholipase A2, group V	R32409	−1.2	PLA2G5	1p36–p34
Contactin 1	R38995	−1.2	CNTN1	12q11–q12
Corticotropin-releasing hormone	R45054	−1.2	CRH	8q13
Wingless-type MMTV integration site family, member 5A	W49672	−1.2	WNT5A	3p21–p14
Carboxypeptidase A3 (mast cell)	T64223	−1.3	CPA3	3q21–q25

Significant gene expression difference between patients and unaffected controls for each gene is $p < 0.05$. The fold change is in the range of ± (1.2–1.4).

decreased in total RNA from lymphocytes by real-time PCR (Fig. 2; − 1.75 fold change; one-tailed *t*-test, $p = 0.043$) which also confirmed the microarray result (− 1.57 fold change; $p < 0.05$). An increase in MDH1 expression (+ 1.26 fold change; one-tailed *t*-test, $p = 0.26$) was not significant but agreed in direction with the microarray results (+ 1.46 fold change, $p < 0.05$). Other microarray gene expression differences within a ± 1.4 fold change that were tested by real-time PCR (APBA3, and PTP4A2) failed to confirm the differences seen in microarray.

4. Discussion

By using a combined approach of a family-based study and microarray analysis, we found two genes, GNAO1 and NPY1R, differentially expressed in transformed lymphocytes of patients with schizophrenia compared to unaffected family members. Both genes were decreased in expression and have been previously implicated in schizophrenia (Table 5).

Alterations in neuropeptide Y (NPY) neurotransmission have been implicated in schizophrenia (Peters et al., 1990). The effects of NPY, a 36-amino-acid protein, are mediated by at least three G-protein-coupled receptors—NPY1R, NPY2R, and NPY5R (for a review, see Redrobe et al., 2002). The NPY peptide is encoded by a single genetic locus at 7p15.1 and is widely expressed throughout the brain. Some postmortem studies have reported alterations in neuropeptide Y levels in the cortex and hippocampus (Kuromitsu et al., 2001; Frederiksen et al., 1991; Gabriel et al., 1996; Iritani et al., 2000). One micro-

Table 5
Significant gene expression differences for schizophrenia and unaffected controls by microarray analysis (Table 3) were surveyed for proximity to linkage and association regions

Gene/cytoband	Association/linkage	Authors
PCDH2/5q31	Linkage to 5q31 in German and Israeli population	Schwab et al. (1997)
	Linkage to 5q21–31 in Irish population	Straub et al. (2002)
NPY1R/4q31.3–q32	Possible evidence of linkage at D4S2361 (4q21) and at D4S2394 (4q28) in Finnish population	Paunio et al. (2001)
APBA3/19p13.3	Spino cerebellar ataxia type 6 linkage with CAG repeat in CACN1A4 (19p13). Study found no association with CAG repeat and schizophrenia in Scottish population.	Breen et al. (1999)
PTP4A2/1p35	1p in Costa Rican population	DeLisi et al. (2002)
	Possible linkage at 1p21 with broadened phenotype	Pulver et al. (2000)
HOXA13/7p15–p14	Translocation found in 1 family t(1;7) (p22; q21) associated with COS	Yan et al. (2000)
MPP3/17q12–q21	Linkage to D17S787 (17q22) in population from England, Wales, Scotland, and Ireland	Cardno et al. (2001)
MDH1/2p13.3	Linkage to 2p13–14 in Irish population	Straub et al. (2002)
	Linkage to 2p13–14 in Palauan population	Coon et al. (1998)
GNAO1/16q13	A missense mutation in GNAO1 (Met129Thr129 in exon 4) has been associated with schizophrenia in Japanese population	Tani et al. (2001)

array study reported that neuropeptide Y gene expression was increased in schizophrenia in the DLPFC (Hakak et al., 2001). The NPY1R and NPY5R genes co-localize to 4q31.3–q32 and are transcribed in opposite directions sharing a common promoter region (Ammar et al., 1996; Herzog et al., 1997; Lutz et al., 1997). The NPY2R gene maps to a distinct locus at 4q31 near the other two receptor genes, suggesting that these receptors arose by duplication. The NPY1R gene is widely expressed throughout the brain and is involved in a number of brain functions including psychomotor activity and feeding. The expression of NPY2R and NPY5R appears to be more localized, in particular to areas underlying feeding behavior. Depending on the cellular type and G protein coupling system, activation of neuropeptide Y receptors can cause either an increase in intracellular calcium or inhibition of cyclic AMP.

There has been little neuropsychiatric research conducted with NPY1R. A single report found that NPY1R mRNA expression is nonsignificantly reduced in DLPFC superficial layers in schizophrenia compared to controls (Caberlotto and Hurd, 2001). Of interest, interactions between dopamine and neuropeptide Y systems have been reported. Regarding linkage studies of schizophrenia, the region containing the neuropeptide Y receptor genes has not been frequently implicated (Table 5). However, using schizophrenia families from an isolated Finnish population, Hovatta et al. (1999) reported suggestive lod scores in the 4q31 chromosome region.

The second gene of interest, guanine nucleotide-binding protein, alpha activating activity polypeptide O (GNAO1) is a member of the subunit family of Gα proteins and has only been recently investigated in neuropsychiatry. GNAO1 is highly expressed in the brain and is enriched in growth cones of neuronal cells and in neonatal brain (Sternweis and Robishaw, 1984). GNAO1 participates in a number of signaling pathways including M2 muscarinic, GABA receptor B, dopamine D2 receptors, opioid, alpha2-adrenergic, somatostatin receptors, and Src/STAT signal transduction.

There are several caveats concerning the microarray study. It is not clear what effect the transformation of lymphocytes may play in alterations that were observed in gene expression in this study. Towards that end, there are no reported studies of lymphocyte cells from the same patients before and after EBV transformation that showed gene expression changes involving the significant alterations found in this study. The lymphocytes from this study's family were transformed with the same virus using the same conditions and subsequently grown under identical conditions. Although it is believed that cell passage might change gene expression, to our knowledge, there are no reports comparing gene expression in normally dividing cells at different pas-

sages when confluent. There are reports of growth factors and differences in cell density and growth rates that do contribute to gene expression (Truckenmiller et al., 2002). However, we have maintained constant growth and harvesting conditions for all cell lines, and the cell lines in this study were grown under identical conditions at the same time. Further experimentation with duplicate cell lines from the same patients and comparing different passages would be helpful to address whether additional biological variation in conducting this type of experiment will play a major role in the outcome of the study. Secondly, the small sample number of genes screened by microarray does not allow us to conclude that the 9 gene differences observed, e.g. NPY1R, MDH1, and GNAO1 gene expression, are the sole differences in this pedigree. We found additional 21 genes that did not meet fold change threshold that might be relevant to the onset or etiology of schizophrenia.

Other factors might be present in brain tissue that cannot be measured in peripheral lymphocyte cells, thus our study represents one approach to help inform future studies of brain tissue using conventional methods such as in situ hybridization. In the present study with a Neuroarray, we have screened for known genes (1128 brain relevant genes) that limit the scope of the results. Currently, we are screening a larger number of transcripts using Affymetrix oligonucleotide arrays which target a larger percentage of the transcriptome with exon specific probe sets to address whether additional candidate genes in this multiplex family can be found. Thus, in the present study with a Neuroarray, we did not screen for genes known to be expressed only in lymphocytes which we would have found with a broader array. Additionally, we did not confirm all gene expression changes observed by microarray with real-time PCR. The incorporation of a radioactive label improves the sensitivity of cDNA microarray to detect changes in gene families while real-time PCR follow-up is generally designed to specifically measure one gene more accurately since there are two specific primers used. A particular gene that is differentially expressed by microarray may fail to replicate by real-time PCR due to the use of different sequences between the techniques. cDNA microarray methods generally are 3′ biased, and we may be measuring some cross-reactive cDNA families.

On the other hand, we have attempted to control for variables such as drug treatment and nutrition, and genetic heterogeneity in a family-based microarray approach of a single cell phenotype. Microarray studies may be useful for additional reasons. A benefit of microarray studies is parallel examination of thousands of molecular markers in biochemical pathways, which might be helpful in pinpointing pathophysiological pathways in schizophrenia. Primarily microarray studies of neuropsychiatric disorders to date have been limited to studies of brain tissue from postmortem samples. Peripheral microarray studies of living subjects may be useful especially if the brain related changes are reflected in the lymphocyte. There may also be trait or state related changes that could be useful in subtyping schizophrenia based upon cellular responses to stress and medications that can be observed in microarray analysis of cell cultures.

Another goal of microarray studies of gene expression is to help prioritize mutation and SNP searches in linkage hot spots (Table 5). This could be important since linkage regions of complex disorders such as schizophrenia are typically rather large (10–30 Mb as a rule) and can contain hundreds of brain related genes (Williams et al., 2002).

The convergent functional genomics model (Niculescu et al., 2000) essentially proposes that disease-specific protein and mRNA changes can be identified through multiple lines of converging evidence. These pieces of evidence can then bring a gene into focus as a candidate gene for further study. For example, following methamphetamine administration in brain, the GRK3 mRNA (G protein-coupled receptor kinase 3) was upregulated in frontal cortex (Niculescu et al., 2000). The GRK3 protein was also decreased in peripheral lymphocytes in bipolar disorder I patients. The GRK3 candidate gene is located on 22q12.1 in a region of linkage for bipolar disorder and schizophrenia. These lines of evidence converge to suggest that GRK3 regulation could be an important candidate gene in bipolar disorder and possible vulnerability to psychosis. In the present study, we have associated schizophrenia and microarray mRNA expression changes in peripheral cells. Two examples of candidate genes in the present study that are present either in an area of linkage or associated with schizophrenia are GNAO1 and MDH1. The MDH1 gene maps to a linkage region on 2p13–14 (Table 3). This region has a genome-wide

lod score of 2.17 with suggestive linkage at DNA marker D2S441 loci (Coon et al., 1998). This marker is approximately 4.3 Mb from the MDH1 gene.

In the convergent functional genomics approach, candidate genes could also be examined in CNS tissue of patients and animals treated with pharmacological agents to test if the candidate genes respond at the protein or mRNA levels. MDH1 gene expression was dysregulated in three studies of schizophrenia brain (Middleton et al., 2002; Vawter et al., 2003; Hakak et al., 2001). Thus, the MDH1 gene could be further studied as there is evidence that antipsychotic drug treatment can affect CNS MDH1 expression (Middleton et al., 2002), MDH1 is a peripheral trait marker in lymphocytes (present study), and MDH1 expression is dysregulated in the DLPFC of patients with schizophrenia (Middleton et al., 2002; Vawter et al., 2003; Hakak et al., 2001).

Another example of the convergent functional genomics approach is GNAO1 which maps to 16q13 and has been associated with schizophrenia in one study examining 175 schizophrenic patients and 172 controls (Tani et al., 2001). A missense mutation in GNAO1 (Met129Thr129 in exon 4) occurred at a higher frequency in patients with schizophrenia; however, the allele frequencies were small (schizophrenia = 0.031 vs. 0.006 in controls) and it is possible this mutation might not play a role in a very large proportion of patients. Nevertheless, there have not been reports of linkage to the 16q region, making it possible that GNAO1 is a gene of small effect that might not be discovered in genome wide linkage studies. In a recent microarray report, GNAO1 was also differentially expressed in the DLPFC of human cocaine dependent subjects (Lehrmann et al., 2003), providing a further connection of GNAO1 gene expression with dopaminergic and adrenergic inputs.

Linkage to schizophrenia is reported in many regions of the genome including replicated findings on chromosomes 5q, 10q, and 13q (Mowry et al., 2000; Baron, 2001). We found genes (FGFR2, AP3S1, BTF3, EFNB2) from these three chromosomal regions that showed small, yet significant, differences between individuals with schizophrenia and unaffected family members in the second tier list (Table 4). Further screening of additional high density families to look for convergent functional genomic findings across multiple families will be of interest to help find candidate genes within these broader linkage regions. Support for chromosome regions that might harbor susceptibility genes can be ascertained through a combination of microarray and traditional linkage studies. Interesting candidate genes from microarray studies might not be located within a linkage region, since gene expression differences might occur as a secondary effect downstream from an actual DNA marker located within the primary linkage region. In the polygenic model of inheritance, multiple genes of small effect that commonly occur could be associated with complex disorders such as schizophrenia. In order to ascertain these multiple genes, measurements of parallel expression of upstream and downstream genes would facilitate finding whether sequence variation in DNA actually produces defective gene regulation.

Thus, by microarray analysis, we may gain some understanding of the functional pathway affected by DNA sequence variations in the promoter region, for example. With the number of genes on a microarray fast approaching the entire transcriptome, we may begin to more fully investigate the co-regulation of multiple genes by blocks of DNA that are associated with complex disorders. Future efforts of screening peripheral candidate gene expression can be a useful tool for possibly discovering and monitoring therapeutic drug targets for intervention in schizophrenia.

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