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Gene expression changes in children with autism

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Abstract

The objective of this study was to identify gene expression differences in blood differences in children with autism (AU) and autism spectrum disorder (ASD) compared to general population controls. Transcriptional profiles were compared with age- and gender-matched, typically developing children from the general population (GP). The AU group was subdivided based on a history of developmental regression (A–R) or a history of early onset (A–E without regression). Total RNA from blood was processed on human Affymetrix microarrays. Thirty-five children with AU (17 with early onset autism and 18 with autism with regression) and 14 ASD children (who did not meet criteria for AU) were compared to 12 GP children. Unpaired *t* tests (corrected for multiple comparisons with a false discovery rate of 0.05) detected a number of genes that were regulated more than 1.5-fold for AU versus GP ($n=55$ genes), for A–E versus GP ($n=140$ genes), for A–R versus GP ($n=20$ genes), and for A–R versus A–E ($n=494$ genes). No genes were significantly regulated for ASD versus GP. There were 11 genes shared between the comparisons of all autism subgroups to GP (AU, A–E, and A–R versus GP) and these genes were all expressed in natural killer cells and many belonged to the KEGG natural killer cytotoxicity pathway ($p=0.02$). A subset of these genes ($n=7$) was tested with qRT-PCR and all genes were found to be differentially expressed ($p<0.05$). We conclude that the gene expression data support emerging evidence for abnormalities in peripheral blood leukocytes in autism that could represent a genetic and/or environmental predisposition to the disorder.

Keywords: Autism; Gene expression; NK cells; Microaarays

Autism is a severe neurodevelopmental disorder characterized by social and communication deficits and ritualistic or repetitive behaviors that appear by age 3. Many etiologies have been suggested for this complex syndrome. Though it is associated with a high degree of heritability, specific genes have yet to be associated with autism. Various other factors have been implicated, including immunological [1–6], neurological [7–9], and environmental [10–13].

Several genomic scans utilizing different cohorts have been performed to identify genes associated with autism or potential susceptibility regions in the genome [14]. These studies have yielded few definitive and reproducible gene associations, in part due to the heterogeneity of the syndrome. It is becoming clear that autism is a complex disorder resulting from the collective actions of multiple genes [15]. Recently, several groups have proposed segregating subjects with autism into subtypes or endophenotypes

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that have measured heritable biological traits, to obtain more homogeneous study populations. This approach has shown promise in identifying significant linkage on chromosome 7 using age of first word as an autism endophenotype [16,17]. Despite these extensive efforts, definitive genetic association or genes that contribute to autism susceptibility have yet to be identified, suggesting the need for an alternative strategy [18].

Genomics, the comprehensive study of genes and their functions, offers an alternative approach to studying autism because of the ability to study global changes in gene expression. To date, the application of microarray technology to neurological diseases has been limited. In a study of autism, postmortem brain tissue from individuals with autism was utilized to show that the glutamate neurotransmitter system is altered in patients with autism [19]. However, the availability of postmortem brain tissues is limited, and the quality of RNA from these tissues can be poor and can potentially erode the robustness of this approach. Therefore, the genomic profiling of peripheral blood cells has emerged as an alternative that is used as a proxy to represent the neural transcriptome.

Gene expression profiling of white blood cells using microarrays has been applied to malignant and immune disorders, including leukemia, lymphoma, systemic lupus erythematosis, rheumatoid arthritis, and many others [20–25]. These studies have helped to identify important diagnostic and prognostic markers as well as potential therapeutic targets. Proof-of-principle blood genomic studies of neurological diseases have been performed in animals [26]. Subsequent studies have demonstrated characteristic blood genomic patterns for acute ischemic stroke [26–28], migraine headache [28,29], Tourette syndrome [28,30], renal cell carcinoma [31], multiple sclerosis [32], benzene exposure [33], trauma [34], and neurogenetic disorders including neurofibromatosis type I, tuberous sclerosis type II, and Down syndrome [35,36]. This approach has been applied to bipolar disorder, for which XBP1 was identified as a genetic risk factor using gene expression profiling of lymphoblastoid cell lines from two sets of discordant twins [37]. These data provide significant support that gene expression analysis derived from circulating blood cells can be utilized to diagnose genetic disorders, identify the genetic and environmental components of the disorders, and help understand the underlying biology.

With regard to autism, studies utilizing cell lines derived from children with autism have shown the potential of this approach. In one study, monozygotic twins with autism of disparate severities were analyzed with gene expression profiling and found to harbor gene expression differences that correlated with the severity of autism and language impairment [38]. In a second report, genomic studies of lymphoblastoid cells differentiated children with autism from typically developing children based upon differences in expression of dopamine- and serotonin-related genes [39]. Further, gene expression profiling differentiated cell lines derived from children with autism and isodicentric chromosome 15 abnormalities from cell lines derived from typically developing children [40]. These data strongly support the idea that genomic profiling of cells in peripheral blood could be particularly promising for providing mechanistic insights and surrogate markers in autism.

Here we report for the first time using genomic profiling of whole blood several novel observations. (1) There are gene expression differences between children with autism and typically developing children. (2) There are gene expression differences between subtypes of autism: autism with regression and early onset autism without regression. (3) A small group of genes expressed predominantly in natural killer (NK) cells is associated with autism.

Results

Microarray data

Genes were identified as being significantly regulated between each group and subgroup using the following criteria: the unpaired t tests corrected for multiple comparisons had a false discovery rate (FDR) of 0.05 (5% false positives) or better; and the fold changes in the regulated genes were 1.5 or more. The combination of the FDR and fold change was used to help ensure the identified genes were likely to be biologically significant. The data sets were then stratified according to diagnostic groups (AU, autism; A-E, early onset autism; A-R, autism with regression; ASD, autism spectrum disorder; and GP, general population). The AU group contained both the A-E and the A-R groups. For the ASD group compared to the AU group and GP group, no genes were identified at the $p < 0.05$ level, respectively. Therefore these ASD comparisons were not utilized for further analysis.

A significant number of genes were identified in the AU groups compared to the GP group. In the AU group compared to the GP group, 55 genes were identified as differentially expressed (unpaired t test, FDR $q < 0.05$, fold change > 1.5) (Supplemental Table 1). For the comparison of A-E to GP group, 140 genes were identified as differentially expressed (Supplemental Table 2). In the A–R group, 20 genes were identified as differentially expressed in comparison to GP (Supplemental Table 3). A comparison of the genes regulated in the A-E and A-R subgroups showed 494 genes (Supplemental Table 4).

In Fig. 1, the three gene lists from the above analysis (AU vs GP, A-E vs GP, and A-R vs GP) are compared to identify a small group of genes shared among the three groups. A total of 12 probe

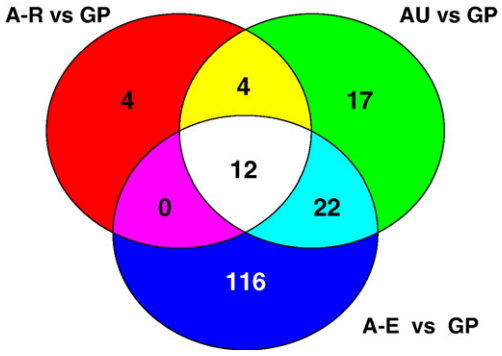

Fig. 1. Venn diagram. Numbers of probe sets significantly regulated for comparisons of the three autism subgroups versus GP (unpaired t test, FDR > 0.05, and fold change > 1.5). This diagram shows the overlap in the numbers of genes for these three comparisons. There were 12 probe sets representing 11 genes for the comparisons of AU vs GP, A-R vs GP, and A-E vs GP.

sets (11 genes) were shared among these three groups (intersection of the Venn diagram and Table 1). These genes were analyzed utilizing DAVID to assess pathway integration. We used 20,254 probes that had expression levels (as identified by GeneSpring) >1 in at least one sample as the background set of genes for the DAVID analysis. This set of genes represents a group of genes that, on average, are expressed in the blood. The KEGG natural killer cell-mediated cytotoxicity pathway was overrepresented in this group of genes ($p=0.02$). In addition, all of these genes are expressed in NK cells and/or $CD8^+$ cytotoxic T cells [28].

Ingenuity pathways analysis (IPA) was used to identify the canonical pathways for each of the three analyses (AU to GP, Table 1; A-E to GP, Supplemental Table 2; and A-R to GP, Supplemental Table 3). Notable pathway overlaps included natural killer cell signaling in all three comparisons, IL-2 signaling and serotonin receptor and dopamine receptor signaling in AU vs GP and A-E vs GP, and retinol and methionine metabolism in the A-E vs GP analysis. The canonical pathways for the three group comparisons are included as supplementary material to this publication (Supplemental Figs. 1–3).

TaqMan confirmation

To confirm the differential expression of selected genes, TaqMan real-time quantitative RT-PCR was utilized. Seven genes, identified as differentially expressed among the autism comparisons compared to typically developing children in the general population, were selected for this analysis. These genes, including SPON2, IL2RB, PRF1, EAT2/SH2D1B, GZMB, CX3CR1, and PAM (Table 1 and Fig. 1), were selected because they were all differentially expressed between AU, A-R, A-E, and GP (Fig. 1) and TaqMan reagents for each gene were available as a validated TaqMan Assay-on-Demand.

Table 1 shows the calculated expression difference for each of the seven genes with calculated p values. The TaqMan data corroborated and validated the direction and magnitude of the expression change of the Affymetrix data for each of the genes common to AU (Table 1). Therefore, although the fold changes observed on the microarrays were modest, the changes were confirmed with quantitative RT-PCR (Table 1).

Table 1
Differential gene expression in autism ($n=35$) compared to typically developing, general population controls ($n=12$)

Gene symbol	Affymetrix GeneChip [a] (fold change)	TaqMan [b]	
		Fold change	p value
PAM	1.86	1.51	0.007
SPON2	1.87	1.86	0.005
IL2RB	1.56	1.35	0.046
PRF1	1.79	1.53	0.027
GZMB	2.01	1.72	0.014
CX3CR1	1.60	1.37	0.006
SH2D1B/EAT2	2.19	1.78	0.011
EDG8	1.99	ND	–
IGFBP7	1.70	ND	–
ZNF145	1.99	ND	–
KIR3DL2	1.58	ND	–

ND, TaqMan not completed for this gene.
[a] All GeneChip fold change results were FDR corrected at $p<0.05$.
[b] TaqMan real-time quantitative RT-PCR was used to confirm the fold changes observed in the Affymetrix microarray analyses. RPL37A and two others were used as a normalization control.

Discussion

The data show differences in gene expression in whole blood of children with autism compared to typically developing children matched for age and gender from the general population. In particular, the gene expression differences of children with early onset autism compared to autism with regression support the possibility that these are two different subtypes of autism. Comparison of ASD to GP did not yield a significant number of genes for either comparison. ASD is a term used for those individuals who do not meet full behavioral criteria in one or more of the autism domains (communication, social, and repetitive behaviors) and therefore individuals in this group have notably different and heterogeneous behaviors. This heterogeneity most likely accounts for the lack of identifying gene expression differences in this group compared to GP. The comparison of all children with autism compared to controls produced a significant number of differentially regulated genes, suggesting that some aspect of "autism" is shared by most of the children sampled. Among these 55 common genes, 11 differentially expressed genes (Table 1) are shared among the three separate comparisons (AU, A-R, and A-E versus GP).

The Venn diagram in Fig. 1 represents these 11 genes shared by the three autism subgroups (AU, A-R, A-E) shown in Table 1. These genes were predominately related to the natural killer mediated cell cytotoxicity pathway. We have previously shown that these 11 genes are all highly expressed in either NK cells or $CD8^+$ cytotoxic T cells from humans [28]. In addition, pathway integration with DAVID demonstrated that the KEGG natural killer cell mediated cytotoxicity pathway was overrepresented in this group of genes ($p=.02$). Using ingenuity systems pathway analysis for functional annotation, classification, and identification of clusters or networks of interrelated genes, we found that in the AU vs GP comparison one of the primary pathways that was regulated was the natural killer cell signaling pathway (Supplemental Fig. 1). The quantitative PCR confirmation of 7 of these genes related to the NK/$CD8^+$ cells provides further confirmation of the microarray results (Table 1). The importance of these findings is that NK cells are a primary, innate defense against viral, bacterial, and parasitic infections or malignant transformation [49]. NK cells bind to infected cells, and if the target cells are MHC Class I negative, they secrete perforins (PRF1—Table 1) onto the surface of the cells to which they have adhered. Perforins permeabilize the cell membrane and permit entry of granzymes (GZMB—Table 1), thereby initiating caspase-mediated apoptosis [49,50]. Similarly, cytotoxic $CD8^+$ T cells are critical to host defense against viruses, intracellular microbes, and tumors by recognizing specific antigens on infected host cells, initiating perforin-induced membrane permeabilization and GZMB (Table 1)—mediated apoptosis [49]. Polymorphisms of the perforin gene affect autoimmunity and lymphoproliferation and produce defective Fas function [51].

NK cells are the predominant white blood cell in the uterus and can mediate pregnancy loss in animals [52]. Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific $CD8^+$ T cells in vivo [53]. IL-2 acts on IL-2 receptors, which are up-regulated in autism (Table 1), and the IL-2 pathway was found to be overrepresented by IPA in AU and A–E vs GP (Supplemental Figs. 1 and 2). IL-2 is known to stimulate proliferation of $CD8^+$ cells and regulates perforin and granzyme gene expression in $CD8^+$ T cells independent of its effects on survival and proliferation [54]. Perforin and STAT4 are both up-regulated in early onset autism (Supplemental Table 2), and perforin is a direct target gene of the transcription factor STAT4 [55].

In addition, SH2D1B/EAT2 (Table 1) is predominantly expressed in NK cells as well as macrophages, B cells, and dendritic cells, and has been postulated to suppress NK cell activity via binding of protein tyrosine phosphatases, inhibitory kinases, or ubiquitin ligases [56]. This is consistent with a report that NK cell activity is reduced in autism [6]. Ylisaukko-oja and colleagues, using fine-mapping linkage analysis, found the highest two-point lod score to chromosome 1q21—q22 (clone D1s484) in a cohort of children with Asperger syndrome [57]. This clone maps 70 kb from SLAMF1 (CD150), one of the key interacting ligands for SH2D1B/EAT2, and less than 2 Mb from the SH2D1B/EAT2 gene itself.

Though the underlying genetic or environmental stimulus for the altered gene expression in the NK and $CD8^+$ cells is currently unknown, the up-regulation of RUNX3 in autism (Table 1) is particularly intriguing since it is a member of the transcription factors that function as scaffolds necessary for interactions of proteins that regulate hematopoiesis and neurogenesis [58]. RUNX3 also regulates proliferation and activation of $CD8^+$ cells and, along with RUNX1, is essential for maturation of $CD8^+$ cells [59,60]. Two other genes regulated in the blood of children with autism, GiMAP6 and DNMT1 (DNA methyltransferase 1) (Supplemental Table 2—early onset autism), play a role in regulating survival of T cells and NK cells [61,62], with DNMT1 being critical for $CD8^+$ cell expansion [62].

The transcription factor RUNX3, moreover, has other functions that also may relate to autism. Abnormalities in RUNX3 function in leukocytes are associated with spontaneous development of colitis and gastric mucosal hyperplasia [63] and could be relevant to autism since a subgroup of children with autism appear to have gastrointestinal abnormalities [64]. RUNX has been implicated in various autoimmune diseases, including rheumatoid arthritis, psoriasis, and lupus [65], and could relate to the subgroup of children with autism who may have an autoimmune disease [66].

RUNX3 also is essential for the target-specific axon path finding of TrkC-expressing dorsal root ganglion neurons [67–69], and its graded activity specifies the laminar termination pattern of sensory axons in the developing spinal cord [70]. Whether RUNX3 plays a role in the forebrain, including language and social regions of brain, that would be relevant for the symptoms of autism is unknown, but merits future study.

In addition to RUNX3, there are several genes expressed in the blood of children with autism that are also expressed in the brain and have specific functions in brain. These genes include semaphorin 4C (SEMA4c; Supplemental Table 3), glutamate dehydrogenase (GLUD1; Supplemental Table 2), endothelial differentiation gene-8 (EDG8; Table 1), *N*-myristoyltransferase-2 (NMT2; Supplemental Table 2), DNA methyltransferase (DNMT1; Supplemental Table 1), and the dihydropteridine reductase gene (QDPR; Supplemental Tables 1 and 2).

SEMA4c and GLUD1 are of particular interest since they relate to glutamate metabolism in brain. A recent study of autism brain by Purcell, Pevsner, and colleagues showed up-regulation of a number of genes related to glutamate neurotransmission [15]. Though SEMA4c and GLUD1 were not specifically identified in that study, they play important roles in glutamate neurotransmission. Semaphorins are a family of proteins that regulate cell motility and attachment in axon guidance, vascular growth, immune cell regulation, and tumor progression. The main receptors for semaphorins are plexins [71]. In brain, semaphorins act as chemorepulsive molecules that guide axons during neural development. SEMA4c, a transmembrane semaphorin, associates with a neurite-outgrowth-related protein, SFAP75 (Norbin), and plays an important role in axon guidance in developing brain [72]. SEMA4c also interacts with a postsynaptic density protein, PSD-95, the major postsynaptic protein associated with the Group 1 metabotrophic and NMDA-subtype ionotrophic glutamate receptor [73]. Immunostaining for SEMA4c overlaps that for PSD-95 in superficial layers I–IV of the neocortex [73] and since SEMA4c binds PSD-95, it would likely play a role in glutamate-mediated neurotransmission [74]. Up-regulation of SEMA4c in blood of children with autism may reflect a similar up-regulation in the brain.

Several other genes unrelated to glutamate neurotransmission were also altered in the blood of children with autism and are also expressed in brain. EDG8 (Table 1) was up-regulated in the blood of children with autism. Endothelial differentiation gene (EDG) proteins are G-protein-coupled receptors activated by the lysophospholipid mediator sphingosine 1-phosphate (S1P) or lysophosphatidic acid. Expression of EDG8/S1P5 in brain is restricted to oligodendrocytes and expressed throughout development [75]. S1P activation of EDG8/S1P5 on O4-positive preoligodendrocytes induced process retraction of the cells via the Rho kinase/collapsin response-mediated protein signaling pathway [75]. Thus EDG8, though it is expressed in blood, could modulate myelin development in brain. This is notable for autism since abnormalities of myelin and the corpus callosum have been reported by a significant number of different groups in the brains of individuals with autism [76–82]. One study identified subgroups of individuals with autism based upon the corpus callosum abnormalities, and one group with early onset had some of the largest changes in the corpus callosum [77].

Beyond the identification of dysregulated genes in autism compared to GP, gene expression differences between the GP and the A-E and A-R groups (Supplemental Tables 2 and 3) were identified. Autism with regression has been reported in 25 to 38% of children and accounted for half of the children in this study using a broader definition described under Materials and methods; it has been thought to represent a subtype of autism [83–87]. These gene expression data provide further evidence to

support the clinical separation of these subgroups [85,86,88–91]. Further, the A-E and A-R comparison provided the largest list of differentially expressed genes (Supplemental Table 4), which supports the idea that early onset autism and autism with regression may be distinct biological disorders with a shared behavioral phenotype—autism. This demonstration of a clinical subphenotype supported by gene expression differences portends the possibility that gene expression profiling might be able to be used to distinguish currently unrecognizable clinical phenotypes of autism that may have different underlying etiologies and outcomes. In the cancer arena, this paradigm has been established such that gene expression analysis is used to stratify patients with previous clinically and pathologically identical breast cancer risk into high-risk and low-risk 5-year survival. The possibilities of identifying subphenotypes in autism, akin to the cancer example, with gene expression analysis are exciting. These gene expression phenotypes may be able to help define the etiology, genetics, and clinical phenotype, as well as the outcome, in autism.

In conclusion, this is the first study to demonstrate that there are gene expression differences in peripheral blood in children with autism compared to the general population (age and gender matched). These genes are predominately associated with NK and $CD8^+$ cells and suggest that dysfunction of these cells may have a role in autism. Additionally, the ability to identify gene expression changes between two subphenotypes of autisms (early onset and regression) suggests that this approach can be used to identify other current anonymous subphenotypes or to validate new clinical phenotypes. Further studies are required to validate these findings. These studies should require that the diagnosis of autism meet criteria on both the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview (ADI) and that known and unspecified phenotypes of autism be utilized and investigated for correlative gene expression profiles. Finally, as a predominant number of genes related CD8 and NK cell types were differentially expressed, cell-type-specific gene expression profiles, as well as functional studies, in subjects may yield more profound and robust gene expression changes.

Materials and methods

Patient population

Children with the diagnosis of autism from one of the regional centers that determine eligibility for state-funded services for persons with developmental disabilities were recruited as cases in the CHARGE study. It is estimated that 75–80% of the total population of children with an autism diagnosis in California are enrolled in these regional centers [41]. The GP controls were randomly sampled from California state birth certificate files and frequency matched to AU cases on age, gender, and areas of residence.

To confirm the referral diagnosis, all children were assessed at the UC Davis MIND (Medical Investigation of Neurodevelopmental Disorders) Institute. Autism or ASD diagnoses were confirmed for all cases using DSM-IV criteria, the ADI-Revised [42], and the ADOS, modules 1 and 2 [43–45]. The ADI-R provides a standardized, semistructured interview and a diagnostic algorithm for the DSM-IV and the ICD-10 definitions of autism [46]. The ADOS is a semistructured, standardized assessment in which the researcher observes the social interaction, communication, play, and imaginative use of materials for children suspected of having AU and ASD. Final autism case diagnosis was defined as meeting criteria on the communication, social, and repetitive behavior domains of the ADI-R and scoring at or above the cutoff for autistic disorder on the ADOS modules 1 and 2. The Social Communication Questionnaire was used to screen for behavioral and developmental characteristics of ASD among the GP. Children who scored above the screening cutoff were fully assessed using the ADI-R and ADOS. For this study, ASD was defined as not meeting the full criteria for autism in one or more domains on the DSM-IV, ADOS, or ADI-R, but meeting criteria for ASD on at least one of two instruments. The developmental and adaptive functions of all children were evaluated with the Mullen Scales of Early Learning [47] and the Vineland Adaptive Behavior Scales [48]. The GP children had scores above 70 on the Mullen Scales of Early Learning and the Vineland Adaptive Behavioral Scales. A developmental–behavioral pediatrician examined each study participant for dysmorphic features or abnormalities on the physical or neurological examinations.

The criteria for autism associated with regression (A–R) were based upon the ADI-R, yes on question 11 or 25 (loss of language or social skills after acquisition), and confirmation by parent interview or separate questionnaire. This is a broader definition than sometimes used, as regression in language was not required for inclusion in the A–R group. Children with early onset autism (A–E) had no evidence of regression in language or social skills on the ADI-R (scores of 0 on both questions 11 and 25). The study protocol followed the ethical guidelines of the most recent Declaration of Helsinki (Edinburgh, 2000) and was approved by the Institutional Review Board of the UC Davis School of Medicine. All subjects enrolled in the study had written informed consent provided by their parents and assented to participate if developmentally able.

Subjects

This case–control study examined 61 children who were enrolled through the MIND Institute into the ongoing CHARGE (Childhood Autism Risks from Genetics and Environment) study at UC Davis that was launched by the Centers for Children's Environmental Health and Disease Prevention Research. The details of the study and study population (National Institute of Environmental Health Sciences P01 ES11269; U.S. Environmental Protection Agency R829388) are further described in a recent publication [13] and described in the supplemental materials and methods.

The sample population included 35 children diagnosed with autism (30 males, 5 females), 14 children diagnosed with autism spectrum disorder (13 males, 1 female), and 12 age- and gender-matched typically developing general population controls with no evidence of ASD (9 males, 3 females). The children with autism were further subdivided, based on clinical characteristics, into 18 children with autism associated with regression (15 males, 3 females) and 17 children with early onset autism (15 males, 2 females).

RNA isolation from whole blood

The RNA was isolated using the PAXgene Blood RNA tubes and kits (Qiagen, Valencia, CA, USA). To mitigate any potential bias in gene expression due to diurnal variation, blood was drawn in the afternoon, preferably between 2:00 PM and 4:00 PM, from all the subjects. RNA quality and purity were analyzed by fiber-optic spectrophotometry using the Nanodrop ND-1000, and RNA integrity was analyzed using the Agilent 2100 Bioanalyzer. All samples consistently achieved A_{260}/A_{280} absorbance ratios of purified RNA exceeding 2.0, and the 28S/18S rRNA ratios were equal to or exceeded 1.8.

Gene expression analysis

Gene expression was assessed on the U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA, USA), with the GC-RMA algorithm implementation within Stratagene's ArrayAssist 4.1 software. The normalized data were then centered relative to the mean of the GP normal control group and exported for further analyses. The data were imported into BRB-ArrayTools version 3.4.0a (http://linus.nci.nih.gov/~brb/download.htm) and Genespring 7.2 (Agilent Technologies Inc., Santa Clara, CA) for data filtering and univariate significance analysis. Significance analysis was conducted using a stringent 1.5-fold unpaired t test with Benjamini–Hochberg FDR correction and filtered for probe sets showing an FDR-adjusted q value of less than 0.05. The Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://niaid.abcc.ncifcrf.gov/), which is used to assess pathway integration for identified genes, was utilized to understand better the differentially

expressed genes. The background set of genes used for the DAVID analyses included only genes that were expressed at values greater than 1 (as identified by Genespring) in at least one of the samples utilized. In addition, data from the various univariate tests were imported into Ingenuity Pathway Systems (IPA version 4.0) for interaction network analysis using both IPA gene networks and canonical pathways.

The microarray data were submitted to the Gene Expression Ombudsmen with Tracking Series No. GSE6575.

Quantitative RT-PCR confirmation of array results

RT-PCR was performed on a selected number of genes as previously described [40]. The genes were chosen from Table 1. Real-time quantitative RT-PCR was performed for each of the identified genes and normalized to a set of three internal housekeeping genes (DDR1, RPL37A, and SMARC2). The housekeeping genes were selected from the GeneChip data in which the coefficient of variation of the expression level between the samples and the groups was low and the mean expression of these was comparable to that of the differentially expressed genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2007.09.003.

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