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Gene expression in cell lines from Propionic acidemia patients, carrier parents, and controls

Kimberly A Chapman^{a,b}, William S. Bush^c, and Zhe Zhang^d

William S. Bush: william.s.bush@Vanderbilt.Edu; Zhe Zhang: Zhangz@email.chop.edu

^aGenetics and Metabolism, Children's National Health System, Washington DC 20010

^bCenter for Genetic Medicine, George Washington University, Washington DC 20010

^cVanderbilt University, Nashville TN

^dCenter for Biomedical Informatics, The Children's Hospital of Philadelphia, Philadelphia, PA

Abstract

Propionic acidemia (PA) is an inborn of metabolism which usually presents with metabolic acidosis and accumulation of 3-hydroxypropionate among other toxins. Examining the gene expression in lymphoblastoid cell lines (LCLs) from PA patients, their carrier parents and age/sex-matched controls at normal glucose and low glucose growth conditions demonstrated differences among and between these groups. Using three-way ANOVA analysis, four DAVID clusters of response were identified of which three of the four clusters showed that LCLs from carrier parents had an intermediate response between healthy controls and PA patients. These differences included changes in expression of cell cycle regulatory genes, mitochondrialrelated genes, and transcriptional regulation. In addition, differences also were observed in expression of genes involved in transendothelial migration and focal adhesion at normal growth conditions when comparing the LCLs from PA patients and controls. These studies demonstrate transcriptional differences between LCLs from PA patients, their parents and biochemically normal controls.

Keywords

Propionic acidemia; propionic aciduria; organic acidemia; gene expression; gene transcription

Corresponding Author: Kimberly A Chapman MD PhD, Genetics and Metabolism, Children's National Medical Center, 111 Michigan Ave NW, Suite 1950, Washington DC 20010, kchapman@childrensnational.org, Phone 202-476-5278, FAX 202-476-5650.

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Appendix 1: Excel spreadsheet showing output identifying Cluster 1

Appendix 2: Excel spreadsheet showing output identifying Cluster 2

Appendix 3: Excel spreadsheet showing output identifying Cluster 3

Appendix 4: Excel spreadsheet showing output identifying Cluster 4

INTRODUCTION

Propionic acidemia (PA, OMIM #606054) is an intoxication-type inborn error of metabolism caused by dysfunction of propionyl CoA carboxylase (EC 6.4.1.3) in converting propionyl CoA to methylmalonyl CoA [1]. This dysfunction results in the diagnostic intermediates of methylcitrate, 3-hydroxypropionate and propionylcarnitine in urine and plasma. Newborn screening has increased the number patients identified early in life with PA and improves outcomes [2]. Patients identified by newborn screening or clinically can have symptoms of metabolic decompensations during episodes of increased metabolic demands and catabolism, such as fever, vomiting, illness, and fasting [1].

Long term complications of PA include cardiomyopathy, skeletal myopathies, metabolic-stroke like episodes [2, 3, 4, 5]. In addition, individuals with PA also have increased risk for infection in that 30–65% have some dysfunction of their immune system [6]. Furthermore, previous studies in related intoxication type disorders such as urea cycle disorders indicate metabolic decompensations are more related to infection than to dietary discretions [7] so immune function is important for overall health.

These studies examine gene expression in cell lines from PA patients, presumably carrier parents and controls. Assaying gene expression is a method to determine which RNAs are produced at a certain time under certain conditions and has been used in several seminal papers. A number of tissues from individuals with inborn errors of metabolism have differences in their overall gene expression profiles compared to healthy controls, including gene expression studies from the brain of a patient with Menkes [8], fibroblasts from patients with mucopolidosis [9], and brains from mice with neuronal ceroid lipofuscinosis [10, 11]. Each of these studies provides insight into underlying disease pathology, especially when the bioinformatic profile confirms known or identifies suspected and unsuspected pathway components [12]. There have not been extensive studies in gene expression in the intoxication metabolic disorders such as PA. Differences in gene expression may identify new approaches and insights into pathophysiology resulting in previously unrecognized therapeutic targets [13].

Our goal in this study was to characterize the gene expression from lymphoblastoid cell lines (LCL) derived from PA patients, carrier parents, and sex- and age-matched healthy controls grown in low glucose (9 mg/dL) and normal glucose growth conditions (120 mg/dl). Decreased glucose was used to force the cell lines to use pathways other than glycolysis for energy production with the goal to model a catabolic state.

MATERIALS and METHODS

Lymphoblastoid Cell Lines (LCLs)

Studies were undertaken with LCLs from 12 clinically diagnosed PA patients, 15 parents of PA patients (presumed obligate carriers) and 12 age- and sex-matched control LCLs from the Centre d'Etrude du Polymorphisme Humain (CEPH) HapMap families (Table 1). All the LCLs (including PA patients, parents, and controls) were commercially purchased from the Coriell Institute for Medical Research cell repository (<http://ccr.coriell.org/>). All the cell

lines were anonymized before we received them and were maintained at 5×10^5 cells/mL in RPMI 1640 (Gibco, Carlsbad, CA) with 15% fetal calf serum (Hyclone Laboratories, Logan UT), 2 mmol per liter L-glutamine (Gibco, Carlsbad, CA), and 100 units per milliliter penicillin-streptomycin (BioWhittaker, Switzerland) (normal conditions).

Low glucose conditions were created by substituting RPMI 1640 with no glucose plus 15% fetal calf serum for glucose-containing RPMI 1640 (Gibco, Carlsbad, CA) plus 15% fetal calf serum 24 hours prior to RNA harvest. Low glucose conditions had a maximum level of glucose of 9 mg/dL (due to the content of glucose in the fetal calf serum), whereas normal conditions contained approximately 120 mg/dL glucose. Low glucose conditions were selected to maximize differences in metabolism since then amino acids and fatty acids would then be the primary sources for energy for these cell lines. PA typically is treated with glucose to reverse catabolism [14]. The goal here was to induce cellular biochemical stress by restricting glucose and inducing catabolism. One expects cell lines from PA patients to create their own intracellular propionic acid metabolites, but experiments by us, not discussed here, using LC-MS and standard organic acid techniques only showed that the lines could produce levels of these metabolites, namely 3-hydroxypropionate and 2-methylcitrate at the level of detection preventing them from being feasible markers for metabolic stress in these studies.

RNA isolation and Hybridization

Cells were lysed using Qiashredder (Qiagen, Valencia, CA) and total RNA was isolated using Qiagen RNeasy purification kits (Qiagen, Valencia, CA). These RNA were labeled and fragmented using the GeneChip Whole Transcript (WT) Sense Target Labeling Assay (Affymetrix, Santa Clara, CA). Resultant cRNA was hybridized to the Affymetrix Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA) in two hybridization groups (batch 1 and 2) due to the number of assays necessary for the study and capacity of the system. Data was submitted to GEO data base (submission series GSE42816).

Microarray analysis and Bioinformatics

Affymetrix Cel files (raw data) from the HuGene1.0st gene expression platform were passed through quality control procedures using the default settings in the Partek analysis suite, after which unsupervised sample clustering was done using Principal component analysis (PCA) of gene expression under normal growth conditions to compare among PA patients, carrier parents (family) and controls. Possible confounding effects of age, sex, batch, and family were evaluated based PCA results and are reported in the result section.

Then samples were split into three groups, controls, patients, and carriers (family), and were coded for two states, normal and low-glucose. Expression data were analyzed using repeated measures analysis of variance using a model containing treatment state (a within subjects factor) and sample group (a between subjects factor). The model also included an interaction term to assess the differences in treatment response between sample groups.

From this analysis, probe sets were selected that had an interaction term significance of $p < 0.0005$, and were mapped to Entrez-gene IDs using Partek annotation files. When multiple probe sets mapped to the same gene, only the most significant probe set was kept. To

identify mechanisms that perturbed in response to glucose between the sample groups, we conducted an analysis using the SPIA package in the statistical software R [15]. Statistical significance of the perturbation effect was determined by permutation testing.

In addition, the difference in the level of RNA at each probe site comparing those grown in low and normal glucose conditions was calculated. Genes identified to differ in expression by 3-way ANOVA model (batch + treatment X group), p value < 0.05 and at least 25% change between treatments (low glucose compared to normal glucose) in one or more of the sample groups were clustered and underwent DAVID analyses to determine their similarities.

qRT-PCR

As a control for microarray quantitation, the same RNA underwent quantitative RT-PCR (qRT-PCR) for 3 selected genes which are involved in the biochemical pathways and identified by the gene expression assays described above to be differentially expressed in a subset of patients and controls. Prior to qRT-PCR, samples were treated with DNase I using the Turbo DNase I assay (Ambion, Austin, TX) according to manufacturer's instructions. cDNA was created using the ABI (Carlsbad, CA) high capacity cDNA kit according to manufacturer's instructions. qRT-PCR was then undertaken on each sample for isocitrate dehydrogenase 2, *IDH2* (Hs00953881_m1) and fumarate hydratase, *FH* (Hs008951618_m1) as target genes relative to *ACTB* (Hs03023943_g1) as the endogenous gene on a 7900HT Fast Real Time PCR system (ABI, Carlsbad, CA). Their amounts were semi-quantified and confirmed microarray results.

RESULTS

Experimental controls and principal component analysis

This study set out to determine 1) whether LCLs from PA individuals were different than those from controls in terms of expression of genes in specific pathways under typical conditions and under stress situations (low glucose) and 2) whether LCLs from PA individuals had global expression differences in their response to low glucose stress compared to control LCLs.

Initially studies were analyzed to identify if the possible large confounding factors, including family, sex, and hybridization group, impacted results using principal component analysis (PCA) All analyses were corrected for hybridization group (batch) since differences were identified (data not shown) between the two hybridization or batches as was expected. Sex and family did not greatly affect analysis (FIGURES 1A and 1B). Although individuals within a family did generally group together, when examined across the entire population, there were no obvious large effects on PCA (FIGURE 1B).

There were differences in gene expression from PA patients, PA carrier parents and normal controls

PCA identified differences between LCLs from PA patients and PA carrier parents, as well as between carrier parents and normal controls after accounting for hybridization group (batch) in terms of their response to normal growth conditions (FIGURE 1C).

There were differences in expression of genes when grown in low glucose and normal glucose conditions

The difference in expression in a particular gene at normal growth conditions compared to low glucose conditions was greater in amplitude of change when comparing the results from cell lines from controls and PA patients (FIGURE 2). Since the difference can be negative or positive in sign, fold change was used to illustrate this result. In other words, cell lines from PA patients when grown at low glucose conditions and normal glucose conditions had greater differences in gene expression (RNA levels) than those from controls for the same gene.

Overall, of all genes analyzed, 1337 genes had differential expression in their response to normal and low glucose when comparing among the groups (affected or PA, carrier parent or control) with at least a $p < 0.005$. The top ten affected genes are listed in TABLE 1. These include *CDC42BPB* and *SF1*. Other notable genes identified in this analysis include *ROCK2*, *PDK3*, *EP400*, *GADD45B*, and *MTHFR*.

ANOVA analysis demonstrated expression differences among cell lines from individuals with PA, their carrier parents, and controls

To further identify differences between the response to low glucose compared to normal glucose in LCLs from individuals with PA, carriers of PA and controls, three-way ANOVA was done. Three hundred-ten genes were identified to differ significantly in their response to low glucose by 3-way ANOVA model (batch + treatment X group), p value < 0.05 and at least 25% change between treatments (low glucose compared to normal glucose) in one or more of the sample groups (LCLs from controls, carrier parents, or PA patients). For each of these genes, expression differences clustered into 4 patterns with very few exceptions. Difference is determined by levels of gene expression in LCLs grown in typical growth conditions minus levels of expression in LCLs grown in low glucose conditions. The directionality of the differences was maintained for analysis.

Each pattern of expression difference identified corresponds to a predominate functional group as characterized by DAVID. Cluster 1 (PA patient with decreased expression difference, control with larger decreased difference, and parents with intermediate) corresponds to mitochondrion, intracellular organelle, histone core, polyunsaturated fatty acids (135 David categories) (listed in SI 1) ($p = 4.35 \times 10^{-7}$); cluster 2 (PA with increased expression difference, control with decreased to no difference, and parent closer to PA levels) corresponds to genes with zinc-finger, CD40L and TNFR2 signaling, regulation of cell cycle, basic-leucine zipper (83 DAVID categories) (SI 2) ($p = 4.33 \times 10^{-4}$); cluster 3 (PA with less expression difference, control with increased expression difference, and parent with intermediate) corresponds to biosynthesis of steroids, oxidoreductase activity,

mitochondrion, (70 categories) (SI 3) ($p = 8.88 \times 10^{-3}$); and cluster 4 (PA with virtually no expression difference, control with large expression difference such that the normal glucose-low glucose shows more expression in the normal glucose treated group and parent closer to PA patient) which corresponds to regulation of transcription, chromatin modification, glucocorticoid R signaling, corticosteroid receptor signaling genes (195 categories) ($p = 6.31 \times 10^{-7}$) (SI 4) (Figure 3). Of the 310 genes, 73 genes compose cluster 1, 82 genes compose cluster 2, 41 genes compose cluster 3, and 114 genes compose cluster 4. Full list of each cluster components are listed in SI 1–4.

Analysis demonstrates additional differences between two groups

In a separate analysis, of the genes with 25% or more different expression between controls and carrier parents, DAVID analyses suggest enrichment of histone genes, immune response genes, and cell cycle genes ($p < 0.05$) suggesting that differences in overall transcription regulation and stress response are perhaps mechanisms to maintain metabolic balance in carriers of this disorder. Among the genes with 25% magnitude change between carrier parents and PA patients, there is an over-representation of genes involved in amino-acid synthesis and chemokines ($p < 0.05$) suggesting that the presence of two mutations instead of one affects the biochemistry of amino acids as well as cellular function more than other pathways. These results provide further avenues for research.

Consequently, PA patients, carrier parents and normal controls have identifiable differences in expression at normal growth conditions and under an increased stressful situation (low glucose). Growth rates were not significantly different between cell lines (data not shown). RT-PCR confirmed expression array results for selected genes of biochemical interest (*FH* and *IDH2*) (data not shown) to ensure correlation in gene expression with the array results. These genes were selected because they were differentially expressed in a subset of patient samples, controls and carriers and are involved directly and indirectly in the tricarboxylic acid cycle (TCA). TCA has been hypothesized to have decreased function in PA. These differential responses seen in TCA cycle intermediates in subsets of cell lines from PA patients do not correlate with their *PCCA* or *PCCB* expression levels (data not shown). However, they do continue to be studied to determine their underlying cause. Given the small numbers in this study (even though it is the largest of its kind), statistical significance in subset analysis is almost impossible to be obtained in the absence of an overly complex model.

Immunogene expression is affected in PA compared to controls at normal growth conditions

Two KEGG pathways were independently identified to have differences in expression comparing the gene expression from LCLs derived from PA patients and controls at normal growth conditions (using probes with p-values less than 0.0005). These pathways both play significant roles in immune function and include the pathways involved in leukocyte transendothelial migration and focal adhesion. The perturbation p-values of these two pathways were $p=0.039$ and $p=0.020$ respectively.

The difference observed in genes involved in leukocyte transendothelial migration was not particularly surprising due to the type of cell line, a lymphoblastoid cell line (LCL) used in this study as well as the clinical features of PA (like immune dysfunction) [6]. The major leukocyte genes that are differentially expressed here included *CTNND1* (catenin delta 1) ($p=0.0004$), *PTK2B* (protein tyrosine kinase 2 beta) ($p=0.0004$), *GRLF1* (glucocorticoid receptor DNA binding factor 1) ($p=0.0001$), and *ROCK2* (Rho-associated coiled-coil containing protein kinase 2) ($p=6E-05$).

Also significantly affected according to KEGG pathway analysis is the focal adhesion pathway, predominately based on differential expression of *FLNA* (filamin A alpha) ($p=0.0001$), *FNI* (fibronectin 1 alpha) ($p=0.0005$), *GRLF1* (glucocorticoid receptor DNA binding factor 1) ($p=0.0001$), *ITGAV* (integrin alpha V) ($p=0.0003$), and *ROCK2* (Rho-associated coiled-coil containing protein kinase 2) ($p=6E-05$).

Clinically immune difficulties are common in this patient population [16] and perhaps this provides additional evidence that leukocyte transendothelial migration and focal adhesion are both necessary for adequate immune and a possible source of the immune dysfunction. To this end, treatment of cultured rat astrocytes with propionic acid has been shown to induce cytoskeletal changes which may be mediated by focal adhesion genes [17].

DISCUSSION

We examined gene expression broadly comparing the expression difference in over 33000 probes among cell lines from individuals with PA (affected), their parents (who are presumably carriers for one mutation in the genes encoding propionyl CoA carboxylase) and “normal controls” and found that there were differences between the expression levels of several genes in the controls’ and PA patients’ cell lines, but also among the controls’, carriers’ and PA patients’ cell lines. To our knowledge, this is the first study of this type to be done in samples from individuals with PA or, for that matter, any intoxication type inborn error of metabolism. In these studies, we identified several major categories of gene responses. They include mitochondrial/Krebs cycle genes, stress response genes and immune genes.

We were struck by the number of essential and fundamental genes and pathways affected on an expression level when a cell line is from an individual with Propionic acidemia or carries a single mutation for this disorder (carrier). Because of the large number of genes with their expression affected, it is difficult to isolate a specific pathway that explains the clinical complications and phenotype due to the small sample size and large number of probes analyzed. Although this study provides a number of interesting targets, its strength is in its ability to illuminate the overall overriding impact of losing function of a single enzyme in a pathway as important as the propionate pathway,

Our findings are similar to several studies in the cancer metabolism field which have illustrated disruption metabolic pathways. In addition, a growing number of mutations in metabolic genes have been shown to lead to cancer [18]. Many of these studies demonstrated an important interaction between intracellular signal transduction and

metabolic pathway energy production. In our cohort, there was an increased activation in several stress response and transcription regulation genes including *CEBPB*, *EIF4A1*, *GADD45*, and *MAT2A*. Expression differences of these stress response genes included genes which control signaling and transcription in the samples from PA individuals. Thus individuals with PA, by the very nature of their inherited enzyme defect, may have altered the balance within the cell in more than the biochemical pathway involved. The impact on transcription in the PA cell lines despite their similar growth to normal cells line was surprising initially, but should not have been given the cancer metabolism literature.

We did examine expression of genes involved in enzymatic reactions related to propionate metabolism and mitochondrial oxidative phosphorylation separately. There were no prevalent pathways involved, but there appeared to be altered response in several metabolic genes including *IDH2* (isocitrate dehydrogenase 2, mitochondrial), *NDUFAB1* (NADH dehydrogenase ubiquinone 1, which has a role in Complex 1), *NDUFB6* (NADH dehydrogenase ubiquinone 6, which has a role in Complex 1), *PDK3* (pyruvate dehydrogenase kinase isozyme 3, which plays a role in phosphorylating pyruvate dehydrogenase for activation and connects TCA to glucolysis), and *SLC25A1* (tricarboxylate carrier protein, citrate transporter). Genes listed above, will be further evaluated in future studies especially given their role in energy production.

Finally we see an expression differences between PA patients and carriers or control individuals in genes encoding transendothelial migration and focal adhesion at baseline growth conditions. One expects genes involved in immunity to be active in these lymphocyte derived lines and could possibly hypothesize *a priori* a possible difference since PA patients have immune dysfunction [16]. This immune dysfunction has manifested itself with an increased risk for infections related to central lines in a very small study [19] as well as by self-report such that 8 (out of 53) individuals reported immunodeficiency at some time during their lifetime [4]. By another study, approximately 30–65% of individuals with PA will have some immune dysfunction including B cell and phagocyte abnormalities [6]. Moreover, there are multiple reports of infections from numerous types of pathogens (for example but not limited to *E. coli*, *S. aureas*, *Klebsiella* and fungus) and death by sepsis [20, 21, 22, 23, 24]. Focal adhesion and transmigration are necessary mechanisms to fight these particular pathogens. Differences in the expression of genes involved in white blood cell migration and adhesion has not been proven to cause disease, but these studies provide tantalizing hints of new lines for exploration of immune dysfunction in PA patients which may assist in the identification and production of therapeutic targets. In fact, ROCK2 appears to be a central hub identified in these studies. ROCK2 inhibitors are presently in production and may provide new avenues for therapeutics for immune dysfunction [25].

One striking finding in these studies is that carriers (parents) may have intermediate expression (or that similar to PA patients) in the DAVID cluster analysis. Although experiment limitations of using LCLs cannot be ruled out, this observation is worth noting since the carrier parents (family) seemed to have intermediate differences in their responses to zinc finger, cell cycle, and mitochondrial related genes (clusters 1, 2, and 3, Figure 3). Historically carriers of autosomal disorders have not been thought to be affected by their carrier status. Initially, the carrier differences from controls (and similarity to PA patients)

were thought to be probably due to familial variation, but PCA indicated that family groups did not significantly affect this analysis.

In sum, we report differences in gene expression in LCLs derived from individuals with PA, carriers and controls that impact the clinically relevant pathways of mitochondrial/Krebs cycle genes, stress response genes, and immune genes. These studies expand the list of possible players in the pathophysiology of PA and so, open new avenues for understanding the disease and developing new therapeutic targets. However, much is still to be done to shape these results into viable therapeutic options.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PA	Propionic acidemia
LCLs	Lymphoblastoid cell lines
CEPH	Centre d'Etrude du Polymorphisme Humain
PCA	principal component analysis

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A

B:

C:

FIGURE 1.

A: Box plot of principal component analysis (PCA) illustrates no obvious large effects according to sex, (Male (blue); female (pink), x axis corresponds to batch #1; y axis corresponds to normal glucose; z axis corresponds to sex). 1B: Box plot of PCA examining impact of family demonstrates no obvious differences (each family all same color, black are controls; x axis corresponds to batch #1; y axis corresponds to normal growth conditions; z axis corresponds to family). 1C: Box plot of PCA illustrating the underlying propionic acidemia status (black are controls, green represent carriers and red illustrates PA patients, x

axis corresponds to batch #1; y axis corresponds to normal treatment; z axis corresponds to whether affected, controls or carriers). Only one hybridization batch is shown here and both hybridization batches show similar findings when analyzed separately (thus only 24 spots instead of 39).

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FIGURE 2.

Graph illustrates the expression difference for an individual gene for control cell lines (x axis) and PA cell lines (y axis) as measured by fold change (1= no change). For the same gene, the PA LCLs have greater differential expression measured by fold change when exposed to low glucose growth conditions compared to the control LCLs. This is true despite similar growth rates for low and normal glucose fed cells, no matter the source of the cells (patient or control). Each dot represents paired results (controls and patients) for the same gene (the size of the dot correlates with the number genes represented). Dots along the

dotted line illustrate the genes with expression changes that are the same for the controls and patients.

FIGURE 3.

Line graphs illustrate the expression differences ($\text{expression level}^{\text{normal glucose}} - \text{expression level}^{\text{low glucose}}$) for patients, family/carriers and controls between low glucose and normal glucose. These maintain the sign (positive or negative) according to which treatment has higher expression, low glucose or normal glucose. Some patterns are similar, but the amplitude of difference from no change (0) dictates directionality accordingly.

TABLE 1

Top 10 differentially expressed genes.

Gene symbol	Gene Description	p-value
CDC42BPB	CDC42 binding protein kinase beta (DMPK-like)	7.43554E-07
KIAA0947	KIAA0947	9.84833E-07
NACC1	Nucleus accumbens associated 1, BEN and BTB (POZ) domain containing	6.74951E-06
CIITA	Class II, major histocompatibility complex, transactivator	7.3147E-06
VPS13C	Vacuolar protein sorting 13 homolog C (<i>S. cerevisiae</i>)	9.47223E-06
PTPRC	Protein tyrosine phosphatase, receptor type, C	9.48736E-06
C17orf71 (SMG8)	Smg-8 homolog, nonsense mediated mRNA decay factor (<i>C. elegans</i>)	2.06297E-05
SF1	Splicing factor 1	2.21111E-05
VPS8	Vacuolar protein sorting 8 homolog (<i>S. cerevisiae</i>)	2.29534E-05
AGXT2L2	Alanine-glyoxylate aminotransferase 2-like 2	2.80461E-05