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Relationship between expression and methylation of obesity-related genes in children

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Abstract

Epigenetic control of gene expression in children remains poorly understood, but new technologies can help elucidate the relationship between expression and DNA methylation. Here, we utilized the nCounter Analysis System to characterise the expression of 60 genes in 69 9-year-old children from a cohort with a high prevalence of obesity. nCounter expression levels ranged broadly (from 3 to over 10000 messenger RNA counts) and were divided into four categories: high (>2000 counts), moderate (200–1000 counts), low (100–200 counts) and marginal (<100 counts). For a subset of five genes (*ADIPOR1*, *PPARG1*, *GSTM1*, *PON1* and *ACACA*) from different expression level categories, we validated nCounter data using reverse transcription-polymerase chain reaction (RT-PCR), and expanded RT-PCR analysis of *ADIPOR1* to include 180 children. Expression data from the two methodologies were correlated for all five genes included in the validation experiment, with estimates ranging from $r_s = 0.26$ ($P = 0.02$) to $r_s = 0.88$ ($P < 5 \times 10^{-6}$). *ADIPOR1* and *PPARG1* nCounter expression levels were negatively correlated ($r = -0.60$, $P < 5 \times 10^{-5}$), and this relationship was stronger in overweight children ($r = -0.73$, $P < 5 \times 10^{-5}$) than in normal weight children ($r = -0.42$, $P = 0.016$). Using methylation data from the Infinium HumanMethylation450 BeadChip ($n = 180$), we found eight CpG sites in *ADIPOR1* and *PPARG* where methylation level was associated with expression by RT-PCR ($P < 0.05$). Hypomethylation of *PPARG* gene body site cg10499651 was associated with increased expression as measured by both RT-PCR and nCounter ($P < 0.05$). We found no statistically significant relationships between either expression or methylation of *ADIPOR1* and *PPARG* and body mass index or waist circumference. In addition to demonstrating the validity of expression data derived from nCounter, our results illustrate the use of new technologies in assessing epigenetic effects on expression in children.

Introduction

Over the past three decades, childhood obesity rates in the USA have been steadily increasing (1–3). Although there is recent evidence that obesity prevalence in young children is declining (4), rates of childhood obesity in Mexican-American children remain considerably higher than in non-Hispanic whites (5–7). It is well accepted that obesity has a heritable component, yet genome-wide association studies have identified few genetic polymorphisms related to obesity (8,9). Although certain variants in genes such as *LEP*, *LEPR*, *POMC*, *PCK1* and *MC4R* can lead to obesity, these variants are rare and therefore explain only a fraction of obesity's observed 40–70% heritability (8,10). It is possible that a portion of this unexplained heritability, an example of the 'missing heritability problem' (11), is due to epigenetic changes that alter the expression of genes involved

in obesity. DNA methylation, the most commonly investigated epigenetic mark, may be a relevant biological mechanism which can influence obesity risk (12,13). DNA methylation refers to the addition of methyl groups to cytosine residues at specific locations in the genome, termed CpG sites, which are clustered in CpG-rich regions known as CpG islands and flanked on either side by larger spans of DNA called shores and shelves (14). CpG methylation can result in altered gene expression, which, in turn, is detectable with expression assays (9,15).

Although quantitative reverse transcription-polymerase chain reaction (RT-PCR) is a standard method utilised in gene expression analysis, there are a variety of powerful new technologies for the assessment of both gene expression and DNA methylation. The NanoString nCounter Analysis System is a digital single molecule

count-based detection system that can assess the expression of many genes in a single multiplexed reaction (16,17). Recently, nCounter has been utilised for transcriptomics in studies investigating a variety of topics including cancer (18,19), infectious disease (20) and immunology (21), but has yet to be employed in the assessment of expression changes associated with obesity in humans. The nCounter Analysis System has a variety of advantages, especially in regards to genetic analyses using banked samples from epidemiological studies. nCounter is relatively affordable and allows for the simultaneous quantification of hundreds of targets in a single small-volume sample. The nCounter method of labelling transcripts with unique colour-barcodes allows for the generation of direct counts of RNA transcript, which is a more readily interpretable data format than the fluorescence values or cycle thresholds (Ct) that are obtained from other expression methodologies. Lastly, nCounter does not rely on a PCR amplification step, meaning it is not vulnerable to variability caused by differences in amplification efficiency between transcripts (22). One recent study reported that nCounter and expression microarray data were highly correlated, especially for genes with significant expression variability (23).

DNA methylation analysis in this study was performed through use of the Illumina Infinium HumanMethylation450 BeadChip. The 450 BeadChip offers genome-wide coverage of methylation, allowing for the interrogation of over 450 000 CpG sites spanning 99% of RefSeq genes (24,25). Many of the CpG sites covered by the 450 BeadChip are located in CpG islands and are thought to be involved in transcriptional control. Although the 450 BeadChip does not achieve the same level of resolution as sequencing-based methylation analysis methods, it is more affordable and generates data that are comparatively easy to interpret (26). The 450 BeadChip has been utilised in epigenetic studies investigating a wide variety of topics, including cancer (20,24,27), arthritis (28), stress (29) and obesity (30).

Here, we employed both the NanoString nCounter Analysis System and the Infinium HumanMethylation450 BeadChip to assess the expression and methylation of 60 genes involved in metabolism and oxidative stress, with a focus on those that may play a role in obesity development. In particular, we investigated the relationships between CpG methylation and gene expression of *ADIPOR1* and *PPARG*, two genes with well-known roles in maintaining metabolic homeostasis. *ADIPOR1*, which encodes one of the major skeletal muscle receptors for the protein hormone adiponectin, plays an important role in glucose and lipid metabolism (31). *PPARG* is alternatively spliced to form two mature messenger RNAs (mRNAs) (*PPARG1* and *PPARG2*) that encode peroxisome proliferator-activated receptor (PPAR) gamma 1 and 2, nuclear receptors that act to control the expression of genes involved in adipogenesis, lipid metabolism and inflammation (32).

In order to assess relationships between expression and methylation, we used banked blood samples from children enrolled in the Center for the Health Assessment of Mothers and Children of Salinas, CA (CHAMACOS) longitudinal birth cohort study (33,34). CHAMACOS children have a particularly high rate of obesity (57% overweight or obese at age 9) (35), even compared to Mexican-American children in the National Health and Nutrition Examination Survey (36). Previously, CHAMACOS studies have shown associations between obesity and genetic susceptibility (37), adipokine hormone levels (38), perinatal factors (39) and environmental exposures (35,40,41). Here, we describe the use of new technologies in cohort studies and examine the relationship between the expression and methylation of obesity-related genes in children.

Materials and Methods

Study population

The CHAMACOS study is a longitudinal birth cohort that aims to assess the health effects of pesticides and other exposures on growth and development in Mexican-American children living in the agricultural region of Salinas Valley, CA (33,34). Mothers were enrolled during pregnancy in 1999–2000 and interviewed when their children were 6 months of age and repeatedly for the next 9 years. Developmental assessments of children, including anthropometrics, were conducted at birth and at the time of each maternal interview. Signed informed consent was obtained from all mothers in the study. The Committee for the Protection of Human Subjects at the University of California, Berkeley, approved all study procedures.

Anthropometric measurements

Children's weights and heights were measured at the 9-year-visit using a calibrated electronic scale (Tanita Mother-Baby Scale Model 1582; Tanita Corp.) and stadiometer, respectively. Child height was measured in triplicate and the average of measurements was used. Child waist circumference was measured at the 9-year-visit with a tape placed above the crest of the ileum. Measurements were recorded to the nearest 0.1 cm after the child exhaled, performed in triplicate and averaged. Body mass index (BMI) was calculated as mass in kilograms divided by the square of height in metres. Children were categorised as normal weight, overweight or obese using the sex- and age-specific BMI cut-offs (85th and 95th percentile, respectively) provided by the 2000 Centers for Disease Control and Prevention child growth data.

Blood collection

Whole blood was collected in BD vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing either heparin as an anticoagulant or no anticoagulant. Aliquots of whole blood, plasma, buffy coat, red blood cells, serum and clot were stored at -80°C until isolation of RNA or DNA.

RNA and DNA isolation

Total RNA was isolated from randomly selected banked whole blood samples from CHAMACOS 9-year-old children using RiboPure Blood RNA Purification Kits (Life Technologies, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, RNA was purified from 100 μl of whole blood from each subject. Following cell lysis in guanidinium-based solution, RNA was extracted using acid-phenol:chloroform. Isolated RNA was purified by solid-phase extraction on glass fibre filter columns included in the RiboPure kit.

DNA was isolated from banked blood clot samples from the same CHAMACOS 9-year-old children using Clotspin Baskets and QIAamp DNA Blood Maxi Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, with minor modifications as previously described (42). Briefly, blood clots were de-clotted using Clotspin baskets, treated with lysis buffer and protease and incubated overnight in a 70°C water bath. DNA was then ethanol-extracted from lysed clots and purified using QIAamp DNA purification columns.

Quality and concentrations of RNA and DNA were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and concentrations were normalized to 20, 1 and 55 $\text{ng}/\mu\text{l}$ before nCounter, RT-PCR or methylation analyses, respectively. RNA quality was also verified using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Purified RNA was

stored up to 2 weeks at -80°C and DNA was stored at -20°C until analysis.

Analysis of gene expression

The nCounter Analysis System (NanoString Technologies, Seattle, WA, USA) was used to simultaneously assess the absolute expression of 60 genes of interest per subject ($n = 80$). Candidate genes of interest were selected based on previous findings in our own research (37,39) or because of their putative or published relationships with obesity or oxidative stress (10,43–45). Purified RNA samples were sent to NanoString Technologies for analysis. Inter- and intra-cartridge replicates were included in the assay. Normalisation of nCounter results was carried out using nSolver Analysis Software Version 1.1 (NanoString Technologies), according to the manufacturer's guidelines. To address both sample and platform sources of variation, RNA counts were normalised using the expression of four reference genes (*ACTB*, *OAZ*, *HUPO*, *HPRT*) and positive spike-in controls included in each sample. Geometric means of positive control and reference gene concentrations were calculated for each lane. Geometric means for all lanes were averaged, and this average was divided by each lane's geometric mean to determine lane-specific scaling factors for both reference gene and positive control expression.

Quality assessment and control were also carried out using nSolver Analysis Software, following the manufacturer's recommendations. Samples were dropped from the dataset if (i) $<75\%$ of counts per field of view were successful, (ii) binding density was >2.25 or <0.05 , (iii) positive control scaling factor was >3 or <0.3 , (iv) reference gene scaling factor was >10 or <0.1 , (v) correlation between known and measured positive control concentrations was less than $R^2 = 0.9$ or (vi) lowest level positive control was under two standard deviations higher than the average of all negative controls. Eleven samples were removed from the dataset due to poor performance, leaving a final subject count of $n = 69$.

To validate expression results from nCounter, relative expression of a subset of five genes of interest (*ADIPOR1*, *PPARG1*, *GSTM1*, *PON1* and *ACACA*) spanning different ranges of expression was assessed *via* RT-PCR in the 69 subjects with nCounter results. The expression of *ADIPOR1*, the gene with the highest level of nCounter expression, was also assessed in an additional 111 subjects to maximise overlap with the 450 BeadChip. Expression of the reference gene *ACTB* was measured for use as an endogenous control. Analysis was completed using QuantiTect Primer Assays and Rotor-Gene SYBR Green RT-PCR Kits (Qiagen), according to the manufacturer's protocol for one-step RT-PCR. Reactions were carried out on a Rotor-Gene 6000 (Qiagen, formerly Corbett Life Science). After verifying amplicon specificity *via* melt curve analysis, Ct were calculated for each gene for each sample. All reactions were performed in duplicate. Internal and negative controls were included in all experiments. Samples or genes with poor coefficients of variance ($<5\%$) between replicates or abnormally high mean Ct (>36) were excluded from analysis.

Site-specific methylation analysis

Site-specific methylation analysis was conducted using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA) on DNA samples from the same 9-year-old CHAMACOS children that were included in expression analysis. Whole genome amplification, enzymatic fragmentation and purification were performed before DNA was applied to the 450 BeadChips according to the Illumina methylation protocol. BeadChips were processed with

robotics and analysed using the Illumina Hi-Scan system. Sample data were extracted using Illumina GenomeStudio Software Version XXV2011.1, Methylation Module 1.9 (Illumina). All samples included in analysis met a quality threshold of having detection P values below 0.01 for 95% of sites. Sites mapping to probe single nucleotide polymorphisms in the Illumina annotation or having P values >0.01 for more than 95% of samples were excluded from analysis. Raw signal intensities were first background corrected, then normalised for colour-channel bias using the All Sample Mean Normalization method as described previously (46). Lastly, the BMIQ algorithm was applied to make interpretation between type I and type II probes comparable (47). Data were expressed as M values, which are calculated as the \log_2 ratio of the intensities of methylated probe to unmethylated probe (48). Negative M values therefore signify that the unmethylated form of a CpG site is more abundant than the methylated form.

Glutathione S-transferase (*GSTM1* and *GSTT1*) genotyping

Study participants ($n = 61$ of the 69 included in nCounter) were also genotyped for *GSTM1* and *GSTT1* homozygous deletion polymorphisms in order to determine whether expression levels were substantially lower in subjects possessing deletions for one or both of these genes. Genotyping was accomplished using the Multiplex Polymerase Chain Reaction Kit (Qiagen) with some modifications (49). As an internal positive control to verify DNA amplification in double null subjects, a 212-bp section of the albumin gene was co-amplified. Gene fragments were simultaneously amplified by using a 96-Well GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA). The null *GSTM1* and *GSTT1* genotypes were detected by the absence of a band at 267 and 434 bp, respectively, after electrophoresis and visualisation on a 3.5% agarose gel stained with ethidium bromide.

Statistical analyses

In order to directly compare results from different gene expression methodologies, relative expression values were computed for both nCounter and RT-PCR. For nCounter data, relative values were calculated by taking the \log_2 of the ratio of normalised expression values of the gene of interest and *ACTB*, a reference gene. For RT-PCR data, the difference in Ct between the gene of interest and *ACTB* is directly interpretable as a 2-fold change, so no transformation is necessary. Therefore, each unit in the relative values from either platform represents a 2-fold difference in expression relative to *ACTB*.

Linear regression and Spearman correlation analysis were used to assess the relationships between relative values for each gene by method of analysis. If both technologies were unbiased estimators of gene expression relative to *ACTB*, linear regression models would be expected to have slopes of one and y-intercepts of zero. Significant deviation from these expected model parameters could suggest bias in either technology.

As a proof of principle, we assessed whether nCounter *GSTM1* and *GSTT1* expression levels differed between subjects with null or wild-type genotypes for these genes, as carriers of null genotypes were expected to have much lower levels of expression. Because expression results from nCounter were not normally distributed, the Wilcoxon rank-sum test was used to test for differences in gene expression between *GSTM1* and *GSTT1* null and wild-type subjects. Subjects were divided into two groups based on their *GSTM1* or *GSTT1* genotypes, and P values were calculated to assess the statistical significance of the differences in expression.

Gene expression results from either nCounter or RT-PCR were compared to methylation M values for *ADIPOR1* and *PPARG* obtained by the 450 BeadChip. First, separate linear regressions were performed to assess the relationship of each individual CpG site with expression as measured by either nCounter or RT-PCR. Reverse stepwise elimination was used to build multivariable linear regression models for assessment of these relationships. Any CpG sites with methylation M values associated with gene expression at a significance level of $P < 0.05$ were selected for inclusion in the final model. Child sex was considered for inclusion as a variable in the model, but did not substantially affect the results. When RT-PCR data were used as the outcome, regression models were adjusted for the expression of the reference gene *ACTB*.

Since *ADIPOR1* and *PPARG1* both contribute to metabolic homeostasis and fat use and storage, Pearson correlation analysis was used to assess the existence of a relationship between \log_2 -transformed expression values from nCounter for these two genes. Relationships between gene expression, methylation and obesity were assessed *via*

Table 1. Subject characteristics

	nCounter mean (SD)	RT-PCR mean (SD)
Total subjects	69	180 ^a
Age (years)	9.1 (0.1)	9.1 (0.2)
% Female	49.3%	53.9%
Anthropometric measures		
BMI (kg/m ²)	20.3 (4.4)	20.7 (4.6)
Waist circumference (cm)	73.3 (11.7)	73.9 (11.8)
Normal weight (<85th percentile)	46.4%	46.7%
Overweight (>85th, <95th percentile)	20.3%	15.0%
Obese (>95th percentile)	33.3%	38.3%
<i>GSTM1</i> and <i>GSTT1</i> genotypes		
<i>GSTM1</i> null ^b	44.3%	–
<i>GSTT1</i> null ^b	14.8%	–

^a $n = 180$ for *ADIPOR1* and 69 for all other genes.

^b $n = 61$ for *GST* genotypes.

several approaches. Both 9-year BMI and categorical overweight status were independently considered for inclusion in the methylation and expression models described above. The relationship between *ADIPOR1* or *PPARG1* expression and obesity was assessed *via* linear or logistic regression, depending on whether BMI, waist circumference or overweight status was used as the outcome variable.

The threshold for significance was set to 5% ($\alpha = 0.05$) for all statistical tests. The Benjamini–Hochberg procedure was used to control the false discovery rate for nCounter and RT-PCR comparisons and for separate linear regressions for each CpG site. All analyses were conducted using statistical software packages Stata Version 12.0 (StataCorp, College Station, TX, USA) and R Version 3.0.2 (www.R-project.org).

Results

Subject characteristics

Subject characteristics for both the nCounter pilot subset ($n = 69$) and the full set of subjects included in *ADIPOR1* RT-PCR ($n = 180$) are presented in [Table 1](#). Similar to 9-year-old obesity prevalence levels in the full CHAMACOS cohort (35), over half (53%) of children were classified as overweight based on BMI above the 85th percentile. Of the 180 children included in this study, 42 and 15% carried homozygous deletions for *GSTM1* and *GSTT1*, respectively.

nCounter expression analysis

[Figure 1](#) shows the range of mean expression levels observed for the 60 genes of interest and four reference genes assessed by the NanoString nCounter Analysis System in whole blood samples. [Table 2](#) summarises four broad categories of observed expression ranges. Only two genes (*ADIPOR1* and *IL8*) were highly expressed, with normalised mean mRNA counts >2000. Thirty-three genes (including *PPARG1*, *GSTM1* and *PON1*) were low to moderately expressed, with normalised mean mRNA counts >100. Twenty-five genes, including *ACACA*, had normalised mean mRNA counts <100 and were comparable with background level and therefore considered marginally expressed.

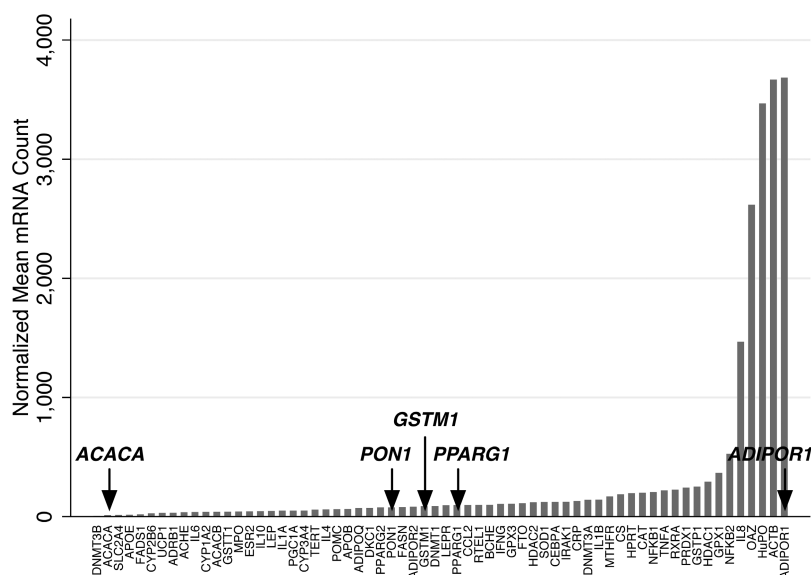


Figure 1. NanoString nCounter expression results. Mean expression values observed for all genes included in NanoString nCounter. Arrows indicate genes validated by RT-PCR.

Comparison of RT-PCR and nCounter expression data

To validate findings from nCounter, expression analysis *via* RT-PCR was carried out for five genes (*ADIPOR1*, *PPARG1*, *GSTM1*, *PON1* and *ACACA*) with a range of expression levels as shown in Figure 1 and Table 2. Summary statistics for RT-PCR Ct and nCounter RNA counts for these five genes are presented in Table 3.

Results of the comparisons between nCounter and RT-PCR methodologies, assessed relative to the reference gene *ACTB*, are presented in Figure 2. Statistically significant correlation was observed between expression data measured by the two expression analysis methods for all five genes, with correlation estimates ranging from 0.26 to 0.88 ($P < 0.05$ for all). Genes with higher expression levels had stronger correlations between methodologies. For example, the strongest correlation was observed for *ADIPOR1* ($r_s = 0.88$, $P < 5 \times 10^{-6}$), the gene for which we observed one of the highest mean RNA counts. Fairly strong correlation was also observed for *PPARG1* ($r_s = 0.62$, $P < 5 \times 10^{-6}$), a gene with low to moderate expression. Somewhat unexpectedly, a low but statistically significant correlation between methodologies was observed for *ACACA* ($r_s = 0.26$, $P = 0.02$), the gene with one of the lowest expression levels of all 60 genes analysed by nCounter. The remaining two genes (*GSTM1* and *PON1*) had intermediate correlation estimates ($r_s = 0.31$, $P < 0.005$ and $r_s = 0.47$, $P < 2 \times 10^{-5}$, respectively) for the comparison between the two assays. All five correlations remained statistically significant after Benjamini–Hochberg adjustment.

In addition to correlation analysis, linear regression was also used to assess the consistency between the two methodologies. Because all values were expressed relative to a reference gene and on a \log_2 scale, regressions were expected to produce slope estimates

of approximately one and y -intercept estimates of approximately zero for each comparison. Slope estimates ranged from 0.14 for *ACACA* to 0.86 for *ADIPOR1*, with all five comparisons having slope estimates lower than one ($P < 0.05$). *PON1* was the only gene for which the y -intercept estimate did not differ significantly from zero ($\beta_0 = -0.95$, $P = 0.43$). The linear models for *ACACA*, *GSTM1* and *PPARG1* had y -intercepts significantly below zero ($\beta_0 = -7.03$, $\beta_0 = -4.85$, $\beta_0 = -2.39$, respectively), while the y -intercept for *ADIPOR1* was greater than zero ($\beta_0 = 4.36$).

Glutathione S-transferase genotypes

Gene deletion variants that inhibit the production of functional protein are common for the phase II detoxification enzymes encoded by *GSTM1* and *GSTT1* (Table 1). Subjects homozygous for these deletion variants are expected to show little to no mRNA expression. In order to determine whether nCounter data were consistent with this expectation, expression results were compared between subjects with or without null (homozygous deletion) genotypes for either *GSTM1* or *GSTT1* (44 and 15% of subjects, respectively). Results for these comparisons are shown in Figure 3. As expected, subjects with the null genotype had substantially lower expression levels for both *GSTM1* (null mean = 54.55, wild-type mean = 114.34, $P < 5 \times 10^{-5}$) and *GSTT1* (null mean = 2.23, wild-type mean = 49.36, $P < 5 \times 10^{-5}$). The variability observed in wild-type subjects is most likely explained by the inclusion of both homozygous and heterozygous subjects in this group.

CpG methylation of *ADIPOR1* and *PPARG*

CpG methylation data from the Illumina Infinium HumanMethylation450 BeadChip included 17 CpG sites in *ADIPOR1* and 21 CpG sites in *PPARG* after quality filtering. M value ranges, summary statistics and gene region information for each CpG site are presented in Figure 4 and supplementary Table 1, available at *Mutagenesis* Online. For *ADIPOR1*, the 450 BeadChip included 6 CpG sites in the gene promoter, 9 in the 5' untranslated region (5' UTR), 1 in the gene body and 1 in the 3' UTR. Mean observed M values ranged from -8.80 to 3.58. For *PPARG*, the 450 BeadChip included 14 CpG sites in the gene promoter, 3 in the 5' UTR, and 4 in the gene body. Mean observed M values ranged from -8.35 to 3.38. Both genes had groups of CpG sites where methylation levels were correlated. Correlation values for sites 2–3 and 14–17 in *ADIPOR1* ranged from $r = 0.49$ to $r = 0.70$, and correlation values for sites 1–2 and 13–21 in *PPARG* ranged from $r = 0.40$ to $r = 0.88$. We observed a general pattern of decreasing methylation in the gene

Table 2. Summary of nCounter expression results

Expression category	Number of genes	Mean count (SD)	Examples
High (>2000)	2	2575.6 (1567.3)	<i>ADIPOR1</i> , <i>IL8</i>
Moderate (200–1000)	12	290.9 (108.0)	<i>NFKB2</i> , <i>GPX1</i> , <i>HDAC1</i> , <i>GSTP1</i> , <i>PRDX1</i> , <i>RXRA</i> , <i>TNFA</i> , <i>CAT</i> , <i>IL1B</i>
Low (100–200)	21	140.6 (33.0)	<i>PON1</i> , <i>GSTM1</i> , <i>PPARG1</i>
Marginal (<100)	25	54.5 (27.2)	<i>ACACA</i> , <i>APOE</i>

Table 3. Summary of RT-PCR and nCounter data

Gene	Function	RT-PCR		nCounter			
		Mean count (SD)	Min	Max	Median count (SD)	Min	Max
<i>ADIPOR1</i>	Receptor for adiponectin, promotes fatty acid breakdown	23.1 (2.1)	20.2	29.8	3204.0 (2398.2)	607.5	10 535.6
<i>ACTB</i>	Beta-actin, reference gene	18.5 (1.1)	16.3	21.9	3317.6 (1140.6)	2087.9	8233.6
<i>PPARG1</i>	Nuclear receptor, stimulates lipid uptake and adipogenesis	24.5 (0.9)	22.3	25.9	59.3 (111.1)	4.7	644.5
<i>GSTM1</i>	Glutathione S-transferase, catalyses phase II detoxification reactions	21.3 (0.5)	20.4	22.5	72.4 (82.7)	2.9	423.8
<i>PON1</i>	Paraoxonase, multifunctional enzyme involved in oxidative stress and organophosphate metabolism	30.9 (1.3)	28.5	35.2	45.3 (105.1)	2.9	563.9
<i>ACACA</i>	Acetyl-CoA carboxylase, participates in fatty acid synthesis	29.4 (1.6)	26.4	33.7	8.3 (10.1)	2.7	57.3

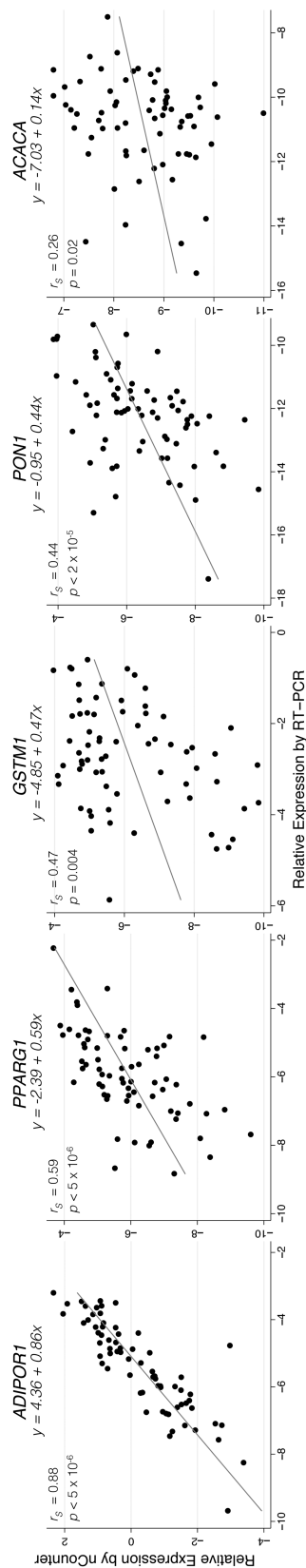


Figure 2. Relationship between nCounter and RT-PCR expression data for five genes. Results from nCounter and RT-PCR are expressed relative to the expression of ACTB, a reference gene, such that each unit represents a 2-fold difference from ACTB expression. Linear regression and Spearman correlation were used to assess the relationship between expression data from each platform.

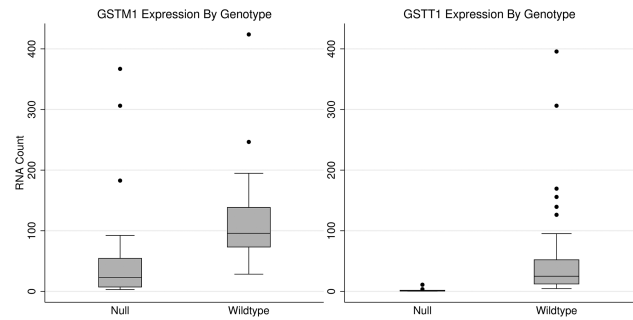


Figure 3. Comparisons of glutathione S-transferase expression by genotype. 'Null' subjects possess homozygous deletions and do not produce functional enzyme. As expected, both *GSTM1* ($P < 5 \times 10^{-5}$) and *GSTT1* ($P < 5 \times 10^{-6}$) show significantly lower gene expression in *GST*-null subjects. P values were computed using the Wilcoxon rank-sum test.

promoter as locations approached the transcription start sites of both genes, with a change to hypermethylation in gene bodies.

Relationship between *ADIPOR1* and *PPARG1* expression and CpG methylation

To analyse the relationship between CpG methylation and gene expression, we first conducted individual linear regressions to assess the relationships between each CpG site in *ADIPOR1* and *PPARG* and either nCounter or RT-PCR expression (supplementary Table 1, available at *Mutagenesis* Online). Next, we used reverse stepwise elimination to build multivariable linear regression models using only those sites that were statistically significantly associated with gene expression (Table 4). Regression models after stepwise elimination showed that three out of 17 *ADIPOR1* CpG sites had methylation levels associated with gene expression as measured by RT-PCR ($P < 0.05$), but no sites were associated with expression measured by nCounter. For *PPARG*, four CpG sites had methylation levels associated with gene expression measured by RT-PCR only, and one CpG site (cg10499651) showed association between methylation and expression measured by both expression assays ($P < 0.05$). Moreover, for this *PPARG* gene body CpG site, both platforms showed the same direction of epigenetic effect, wherein hypermethylation was associated with reduced expression (lower RNA counts in nCounter and higher Ct values in RT-PCR).

Gene expression, CpG methylation and obesity parameters

We observed a strong negative association between *ADIPOR1* and *PPARG1* nCounter gene expression ($r = -0.60$, $P < 5 \times 10^{-5}$). Interestingly, this correlation was substantially stronger in overweight children ($r = -0.73$, $P < 5 \times 10^{-5}$) than in normal weight children ($r = -0.42$, $P = 0.016$) (Figure 5).

Both 9-year child BMI and categorical overweight status were separately considered for inclusion in the expression and methylation stepwise models described above, but showed no evidence of statistically significant association. Using linear and logistic regression, we did not find any associations between *ADIPOR1* or *PPARG* expression or methylation and BMI, waist circumference or odds of being overweight at 9 years of age (data not shown).

Discussion

nCounter RNA expression data were compared to Ct data generated by RT-PCR, the historically standard method, for five genes with a wide

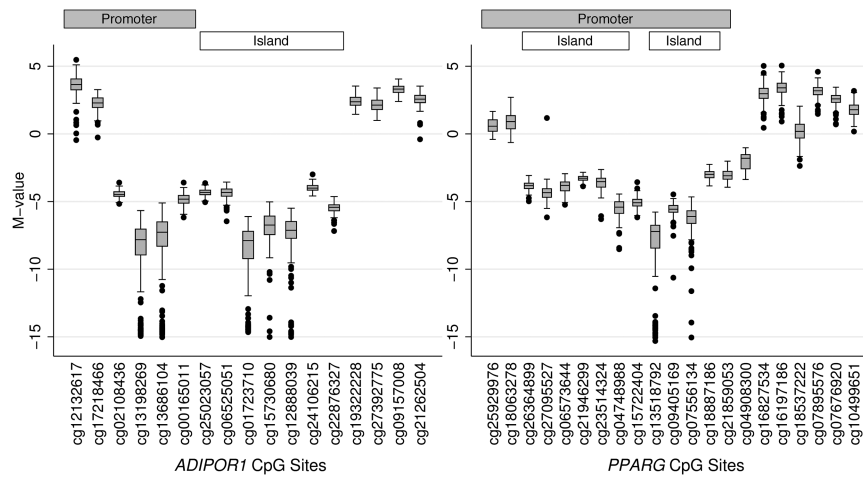


Figure 4. Range of observed methylation *M* values for CpG sites in *ADIPOR1* and *PPARG*. CpG site box plots are ordered according to their location in the genetic sequence. Horizontal bars indicate whether individual CpG sites are located within gene promoters or CpG islands.

Table 4. Association between CpG methylation and expression

CpG site	β	<i>P</i> value	Regulatory region
<i>ADIPOR1</i> nCounter expression			
No sites significantly associated with nCounter expression			
<i>ADIPOR1</i> RT-PCR expression ^a			
cg06525051	0.60	0.019	5' UTR, island
cg15730680	-0.18	0.012	5' UTR, island
cg09157008	0.69	0.034	Gene body
<i>PPARG1</i> nCounter expression			
cg10499651	-59.25	0.049	Gene body
<i>PPARG1</i> RT-PCR expression ^a			
cg04748988	0.37	0.020	Promoter, island
cg13518792	0.08	0.016	Promoter, island
cg21859053	-1.38	0.013	Promoter, shore
cg04908300	0.87	0.009	5' UTR, shelf
cg10499651	0.53	0.024	Gene body

^aNote that higher Ct values and positive β estimates in RT-PCR are indicative of lower expression.

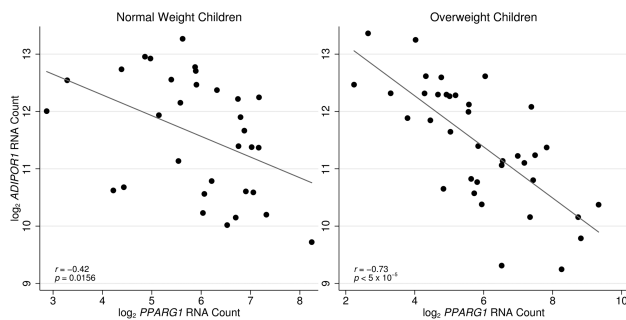


Figure 5. Relationship between *ADIPOR1* and *PPARG1* nCounter expression by overweight status. A strong negative correlation was observed between *ADIPOR1* expression and *PPARG1* expression ($r = -0.60$, $P < 5 \times 10^{-5}$). This relationship was substantially stronger in overweight children ($r = -0.73$, $P < 5 \times 10^{-5}$) than in normal weight children ($r = -0.42$, $P = 0.0156$).

range of observed expression levels. For all genes tested, we observed statistically significant correlations between results ($r_s = 0.26-0.88$), with much stronger correlation estimates for genes with higher expression in blood. Parameters of regression models indicate that although

nCounter and RT-PCR results are correlated, one or both platforms may produce somewhat biased results. For example, RT-PCR is vulnerable to biases resulting from differences in PCR amplification efficiencies, which is not an issue in nCounter (22). However, the results of our RT-PCR validation of nCounter data suggest that both methodologies can be reliably used to compare gene expression between subjects within cohort or case-control studies (i.e. to determine which subjects are ‘high’, ‘medium’ or ‘low’ expressers). We also showed that subjects with homozygous *GST* deletion genotypes had substantially lower nCounter expression levels than wild-type subjects. The high expression levels observed for some *GSTM1*-null subjects may be attributable to compensation by another gene in the *GST* superfamily. For example, Bhattacharjee *et al.* (50) have reported that *GSTM2* is 99% homologous to *GSTM1* and capable of compensating for low enzyme expression in individuals with the *GSTM1*-null genotype. Altogether, our results augment existing nCounter validation efforts using both expression microarrays and RT-PCR (16,23), and confirm nCounter’s potential for use in population studies.

We focused our analyses of gene expression and CpG methylation on *ADIPOR1* and *PPARG*, two important obesity-related genes. Adiponectin receptor 1 is one of the major receptors for the protein hormone adiponectin, and plays a significant role in glucose and lipid metabolism (31). *PPARG* is one member of a family of three PPARs that control the expression of a network of genes involved in adipogenesis, lipid metabolism, inflammation and the maintenance of metabolic homeostasis (32). Animal studies have demonstrated that upregulated expression of *ADIPOR1* leads to increased AMPK and PPAR α activation, thereby reducing gluconeogenesis, increasing levels of fatty acid oxidation and ameliorating diabetes (51,52). Conversely, the nuclear receptors PPAR γ 1 and PPAR γ 2 act to promote adipogenesis and lipid uptake when an excess of fat is present (53).

Consistent with the biological roles of these two genes, we observed a significant inverse relationship between *ADIPOR1* and *PPARG1* expression. Interestingly, this negative correlation was substantially stronger in overweight children than in normal weight children, possibly indicating that metabolism in overweight children is less balanced and more strongly polarised towards either lipid uptake or fatty acid oxidation. To our knowledge, this relationship between *ADIPOR1* and *PPARG1* expression in overweight children has not been previously reported. However, it has been shown that obese adults tend to have lower levels of *PPARG1* expression (54)

and higher levels of *ADIPOR1* expression than non-obese individuals, possibly to compensate for the reduced levels of plasma adiponectin observed in obesity (55). Although it is not yet established in the literature, it is also possible that genetic polymorphisms (such as the pro12ala allele in *PPARG*, present in 10% of Mexican-Americans) may affect both obesity risk and the expression of these two genes (56–59). Since the expected number of variants would be low given our sample size, we did not genotype *ADIPOR1* and *PPARG* polymorphisms in this study.

We were also interested in assessing whether expression or methylation of *ADIPOR1* and *PPARG* were associated with obesity parameters such as BMI and waist circumference. It has already been established that changes in DNA methylation can affect the expression of genes involved in adipogenesis and metabolism, suggesting epigenetic dysregulation may be an essential mechanism for obesity development in children (60–62). In our study, however, we did not observe statistically significant associations between DNA methylation in these two genes and BMI, waist circumference or odds of being overweight at 9 years of age.

Finally, we demonstrated that specific CpG sites may be capable of altering *ADIPOR1* and *PPARG* transcription. Using nCounter, RT-PCR, and the 450 BeadChip, we identified three CpG sites in *ADIPOR1* and five CpG sites in *PPARG* that were significantly associated with gene expression. These sites spanned a variety of regions, including CpG islands, shores, 5' and 3' UTRs and gene bodies. Six of these CpG sites were associated with lower expression and two with higher expression. Though CpG island methylation is often considered to exert significant control over transcription, recent results from published studies indicate that CpG methylation in other gene regions may affect expression in unique ways (63–66), and the relationship between methylation and expression appears to be highly contextual (14). For one of the CpG sites in *PPARG*, we were able to validate our results across both nCounter and RT-PCR platforms. This particular CpG site is located in the gene body of *PPARG*, a gene region that has previously been shown to participate in splicing control (14). It is possible that this CpG site could be involved in *PPARG* splicing, since *PPARG1* mRNA is one of two possible alternatively spliced *PPARG* gene products (32).

There are several strengths to the design of our study. It is one of few that directly assess the relationship between site-specific DNA methylation and gene expression, an area of epigenetics that is still poorly understood. Additionally, studies of this type seldom investigate minority or youth populations such as the CHAMACOS cohort. We also took advantage of data on the presence or absence of null genotypes for *GSTM1* and *GSTT1* in our subjects to assess the relationships between these genotypes and nCounter expression levels. Lastly, we further validated nCounter expression results using RT-PCR, the standard method for expression analysis.

Limitations of our study include relatively small sample size and selective analysis of CpG sites by the 450 BeadChip. Other CpG sites not interrogated by the 450 BeadChip may also regulate gene transcription or show association with obesity parameters; these sites can be assessed by targeted sequencing in future studies. The lack of observed association between methylation and obesity parameters in our study could also be attributed to the existence of longitudinal effects of methylation, important effects of other obesity-related genes or characteristics of this unique minority cohort. Additionally, we conducted this cohort study using DNA and RNA from blood because it is a readily accessible tissue and relatively non-invasive to collect, but we are mindful that methylation and expression variability may be tissue-specific (13,67,68). However, a wide variety of studies have

successfully used blood as a biomarker of altered methylation patterns (69,70), and some analyses suggest that changes to the blood methylome can reflect broader changes across other tissues (71,72).

Conclusion

In this study, we employed two methods of gene expression analysis and a site-specific DNA methylation assay to investigate the relationship between methylation and expression in Mexican-American children. Using nCounter, RT-PCR and the 450 BeadChip, we reported several associations between DNA methylation and expression for the obesity-related genes *ADIPOR1* and *PPARG*, and also showed that the expressions of these two genes are inversely correlated, especially among overweight children. Our findings suggest that individual CpG sites may be capable of either up- or down-regulating gene expression, and that sites in both gene promoter and gene body regions may be important in controlling transcription. These findings may contribute to understanding how DNA methylation may act through gene expression to influence metabolism and obesity development.

Supplementary data

Supplementary Table 1 is available at *Mutagenesis* Online.

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