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POTENTIAL EPIGENETIC DYSREGULATION OF GENES ASSOCIATED WITH MODY AND TYPE 2 DIABETES IN HUMANS EXPOSED TO A DIABETIC INTRAUTERINE ENVIRONMENT: AN ANALYSIS OF GENOME-WIDE DNA METHYLATION

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Abstract

Objective—The aim of this study is to investigate the potential role of DNA methylation in mediating the increased risk of developing type 2 diabetes in offspring of mothers who had diabetes during pregnancy.

Materials and Methods—Peripheral blood leukocytes were collected from non-diabetic Pima Indians who were either offspring of diabetic mothers (ODM; n = 14) or offspring of nondiabetic mothers (ONDM; n = 14). The two groups were matched for age, sex, age of mother, and fraction of Pima ethnicity. Differentially methylated regions were determined using a MeDIP-chip assay on an Affymetrix Human Tiling 2.0R Array. Data were analyzed using the model based analysis of tiling arrays (MAT) algorithm, and 4883 regions overlapping with putative promoters, were identified as differentially methylated, having met an empirically derived threshold (nominal $p < 0.0077$). The list of genes with differentially methylated promoters were subjected to KEGG pathway analysis to determine canonical metabolic pathways enriched by these genes.

Results—Pathway analysis of genes with differentially methylated promoters identified the top 3 enriched pathways as maturity onset diabetes of the young (MODY), type 2 diabetes, and Notch signaling. Several genes in these pathways are known to affect pancreatic development and insulin secretion

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Author contributions

Data were acquired by MCD, VO, WCK, and RLH; analyzed by MCD and RLH; and interpreted by MCD, WCK, CB, LJB, and RLH. MCD designed the figures and wrote the paper with advice from WCK, CB, LJB, and RLH.

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Disclosure statement

The authors have no competing financial interests to declare.

Conclusions—These findings support the hypothesis that epigenetic changes may increase the risk of type 2 diabetes via an effect on β -cell function in the offspring of mothers with diabetes during pregnancy.

Keywords

in utero; maternal diabetes; immunoprecipitation; microarray

INTRODUCTION

Studies in Pima Indians were the first to provide evidence that individuals whose mother had type 2 diabetes during their pregnancy are at much greater risk for developing diabetes compared with individuals whose mother was nondiabetic during the pregnancy [1,2]. Studies of Pima siblings discordant for intrauterine exposure to diabetes have shown that this risk is mediated by the intrauterine environment *per se* in addition to the risk due to inheritance of diabetes-susceptibility alleles [3]. Diabetes in Pima Indians, regardless of age of onset, appears to be entirely type 2, based on absence of absolute insulin dependence, rarity of ketoacidosis, and lack of antibodies to islet cells or glutamic acid decarboxylase [4,5].

Metabolic studies of offspring exposed to a diabetic intrauterine environment have found impaired insulin secretory function as opposed to impaired insulin action, indicating a defect in insulin secretion underlying the increased risk for type 2 diabetes [6].

Epigenetic mechanisms, such as DNA methylation, are strong candidates for mediating the effects of exposure to the diabetic intrauterine environment. However, there is little information on specific DNA methylation patterns that may be associated with exposure to diabetes *in utero*. Using genome-wide tiling microarrays, we studied differences in methylation of human peripheral blood DNA from nondiabetic Pima Indians born to mothers with and without type 2 diabetes during pregnancy.

RESEARCH DESIGN AND METHODS

Study subjects

Subjects were participants in a longitudinal study of diabetes in the Gila River Indian Community, where most of the residents are Pima Indians. Community residents 5 years of age were invited to a health examination every two years [7]. These examinations included a 75-g oral glucose tolerance test (OGTT) and diabetes was diagnosed according to 1997 American Diabetes criteria (fasting plasma glucose ≥ 7.0 mmol/l or 2-hour post-load plasma glucose ≥ 11.1 mmol/l) [8] or if the disease had been diagnosed during routine clinical care. The same test procedure and diagnostic criteria were applied in nonpregnant participants and during pregnancy. Peripheral blood leukocytes (PBL) were collected and stored frozen as pellets. For pregnant women, glycemia was measured either at a biennial examination or a third trimester OGTT. The longitudinal study was approved by the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

For the present study, 14 unrelated subjects were selected who were offspring of diabetic mothers (ODM) in that the mother had documented diabetic levels of hyperglycemia at an examination during the 9 months preceding the child's birth (Table 1). Thirteen of the mothers had been diagnosed with diabetes at an examination prior to the pregnancy OGTT. All offspring were at least 50% American Indian heritage, had a PBL pellet available from a nondiabetic biennial examination, and had HbA1c <6.5%. For comparison, 14 unrelated nondiabetic individuals were selected who were offspring of nondiabetic mothers (ONDM) in that the mother had documented normoglycemia (2-hour post-load glucose <7.8 mM) during the 9 months prior to the child's birth and had a non-diabetic examination at least one year after the child's birth. Fourteen ONDM were selected within strata of sex, age group (10-14, 15-24, 25-34 years), age of mother at delivery (20-29, 30 years) and ethnicity (full- or partial-heritage American Indian), resulting in ten strata each containing 1 to 4 ODM and a matching number of ONDM.

Methylated DNA Immunoprecipitation (MeDIP)

DNA was immunoprecipitated (IP) with a mouse monoclonal antibody against 5-methylcytosine (clone 10G4, Zymo Research #A3001-50) and recovered using magnetic beads (Dynabeads M-280 sheep anti-mouse IgG, Dynal Biotech #112.01) [9]. A portion of the sonicated DNA (Input) was left untreated and used as control. DNA was amplified and cleaned using the GenomePlex Complete Whole Genome Amplification Kit and GenElute PCR Clean-Up Kit (Sigma). For microarray experiments we interrogated IP and Input DNA against the Genechip Tiling 2.0R Array, on a GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, CA).

Statistical and Bioinformatic analysis

Data files were imported into the Partek Genomic Suite (PGS) software. We pre-normalized data using quantile-normalization and corrected for background using the Robust Multichip Averaging algorithm before conversion to log₂ [10]. Baseline normalized values were obtained by subtracting the mean signal for the Input array from the matching IP array for each probe. To detect enriched regions, data were analyzed using the model-based analysis of tiling arrays (MAT) algorithm [11] as implemented in PGS. For each probe, standardized intensities were compared between ODM and ONDM in a 3-way ANOVA model fit by the method of moments [12], with control for stratum and age of offspring (to account for residual confounding within strata), resulting in a t-statistic for each probe [13]. The mean of these probe-level t-statistics was then calculated for regions constructed from 600 bp sliding windows across the genome, with the restriction that each window contains 10 probes. (We also analyzed the trimmed mean, which excludes the 10% of probes with the highest values and the 10% with the lowest values and obtained similar results.) To enable analyses that use information for all regions, the p-value threshold for inclusion in subsequent analyses was set at 1.0. The mean of the t-statistics and the number of probes analyzed are then used to assign a MAT score and a corresponding p-value for each region. The algorithm then merged nearby regions (within 300 bp) and assigned them the MAT score and p-value associated with the most strongly scoring window in each region. The magnitude of the MAT score reflects the relative methylation of one group compared with the other. As presently constructed, regions with positive MAT scores are hypermethylated

in ODM compared with ONDM, while regions with negative MAT scores are hypomethylated in ODM compared with ONDM.

Analyses were restricted to regions that overlapped with putative promoters (defined as the region between a point 2000 bp upstream and one 500 bp downstream of each transcription start site in RefSeq Build 36). This resulted in 30,201 unique regions in 21,332 promoters across the genome. Since some regions can be assigned to more than one gene and some genes are covered by more than one region, a total of 33,995 gene-region pairs were analyzed. The false discovery rate (FDR) procedure [14] was used to account for multiple statistical comparisons. To account for distributional irregularities (e.g. due to small sample size, lack of normality or taking the strongest MAT score) the expected distribution of p-values under the null hypothesis was generated by permutation for an empirical FDR calculation; exposure status (i.e. ODM vs. ONDM) was randomly permuted within strata and 100 permutations were analyzed to generate the expected distribution. The mean MAT score across all regions was taken as a measure of whether global methylation was different between ODM and ONDM; statistical significance was determined by comparing this value with its standard error, estimated by a jackknife procedure in which each stratum was omitted [15]. In addition relative enrichment (RE) of the observed cumulative p-value distribution compared to the expected distribution, computed from the permutations, was calculated to determine a threshold for differential methylation. If $F_{\text{obs}}(p_t)$ represents the observed cumulative proportion of p-values < a given threshold p_t and $F_{\text{exp}}(p_t)$ the cumulative proportion expected, then $RE = [F_{\text{obs}}(p_t) - F_{\text{exp}}(p_t)] / F_{\text{exp}}(p_t)$. The p-value at which RE was maximized was taken as a threshold to deem regions differentially methylated.

Fisher's exact test was used to determine whether the proportion of differentially methylated gene regions among established diabetes or obesity genes was different from the proportion among all other genes on the array. We thus tested genes established as associated with monogenic maturity-onset diabetes of the young (MODY) [16-18] or established through genome-wide association studies as associated with type 2 diabetes or BMI [19-22]. To determine whether genes in canonical metabolic pathways were differentially methylated in ODM vs. ONDM, differentially methylated genes were analyzed with the Functional Annotation tool of the Database for Annotation Visualization and Integrated Discovery (DAVID) version 6.7 [23,24] KEGG pathways [25] were analyzed to determine if they were enriched for differentially methylated genes compared with the "background" of all other genes on the array. Pathways with an enrichment p-value (EASE score) <0.05 were considered significantly enriched.

RESULTS AND DISCUSSION

There was a global tendency for ODM to be hypomethylated relative to ONDM, as reported in islets from human donors with type 2 diabetes relative to non-diabetic donors [26], but this difference was not statistically significant (mean MAT score = -0.26, SE = 0.19, $p = 0.17$). Figure 1A shows the logarithm of the p-value plotted against the FDR for all 33,995 regions that overlap with a known gene promoter region. None of the regions had $FDR < 0.05$. The maximum relative enrichment of the observed vs. the expected cumulative distribution of p-values occurred at $p < 0.0077$ (Figure 1B). Considering this p-value as a

cutoff, 14.4% ($n = 4883$) of gene regions show differential methylation between ODM and ONDM. Due to overlapping sequences, these 4883 gene regions represent 4035 unique regions; their chromosomal locations are shown in Supplemental Figure S1. The 20 regions showing the most significant differences in methylation between ODM and ONDM are given in Table 2; all 4883 regions are shown in Supplemental Table S4. Although it is likely that many of these represent truly differentially methylated regions and many represent statistical artifact, the high FDR values indicate that these cannot be confidently distinguished among individual gene regions with the present sample size. Subsequent analyses were performed to identify pathways enriched for differentially methylated regions.

The 4883 differentially methylated gene regions constitute 4501 unique genes and bioinformatic analyses comparing these genes with the 21,332 “background” genes on the array identified 13 KEGG pathways [25] that were significantly enriched for differentially methylated regions (Table 3). The pathways containing the largest proportion of genes differentially methylated between ODM and ONDM are: MODY, type 2 diabetes mellitus and Notch signaling (Figure 2).

KEGG pathways were constructed based on biochemical relationships and they do not contain all known MODY genes or most genes established as associated with type 2 diabetes. Direct classification of the 13 known MODY genes resulted in 17 unique regions of which six (35.3%) are differentially methylated (Supplemental Table S1). Similarly, classification of 60 type 2 diabetes genes, primarily identified in genome-wide association studies, (Supplemental Table S2) resulted in 103 regions of which 26 (25.2%) are differentially methylated. The proportion of differentially methylated genes for either MODY or type 2 diabetes is significantly greater than that observed for all other genes ($p = 0.026$ for MODY and $p = 0.004$ for type 2 diabetes). In contrast, the proportion of differentially methylated regions from 32 established obesity genes is not statistically significantly different from all other genes (10 of 54 regions, $p = 0.435$, Supplemental Table S3).

These analyses show that regions in promoters of genes involved in MODY and type 2 diabetes are more likely to be differentially methylated between ODM and ONDM than are other genes. Genes associated with MODY and type 2 diabetes, as well as genes in the Notch and WNT signaling pathways, have been implicated in pancreatic development, maintenance of β -cell glucose responsiveness and insulin secretion. Thus, the present results are consistent with the concept that exposure to hyperglycemia *in utero* affects methylation of genes involved in β -cell function, and that this in turn results in the high risk of diabetes in exposed individuals. Although sequence variation in some of these genes can cause diabetes (e.g. MODY), our data do not demonstrate directly that the observed altered methylation patterns cause diabetes in those exposed to diabetes *in utero*. All subjects were nondiabetic when examined, but the observed epigenetic profile could be the secondary consequence of a pre-diabetic metabolic state rather than a primary cause of diabetes. The statistically significant differences in BMI and HBA1c observed between ODM and ONDM may reflect such differences in metabolic state. We did not adjust for these differences, since with the current cross-sectional design it is difficult to determine whether they mediate the

epigenetic effects or whether the epigenetic effects are secondary; longitudinal studies may be required to distinguish these possibilities.

This study utilized DNA from peripheral blood that might not represent methylation patterns from all other cells. The results from the Dutch Famine Study showed that genome-wide methylation do not support an effect of pre-natal famine on adult global DNA methylation levels in white blood cells. As the same cohort showed pre-natal famine environment has been associated with a persistent decrease in DNA methylation of the *IGF2* gene, their findings suggest that the effects of prenatal exposure on DNA methylation may be locus-specific, rather than on global DNA methylation in adulthood [27,28].

Genome-wide DNA methylation changes associated with type 2 diabetes have been previously shown in peripheral blood, and were observed prior to disease manifestation indicating an early onset methylation risk profile for later impaired glucose metabolism and subsequent diabetes [29]. Furthermore, a comparison of differential methylation of different internal tissues and blood showed that a systematic annotation of tissue-specific differentially methylated regions of internal tissues showed a correlation to the DNA methylation measured in easy accessible tissues, such as peripheral blood. Despite the predominance of tissue differences, inter-individual differences in DNA methylation in internal tissues were correlated with those for blood for a subset of CpG sites in a locus- and tissue-specific manner [30].

DNA methylation can be mitotically stable over time, producing long-term changes in gene expression. The present study suggests that changes in DNA methylation of genes involved in pancreatic development and insulin secretion may result in epigenetic dysregulation of these genes, which may mediate the increased risk of diabetes in individuals exposed to a diabetic intrauterine environment.

Further studies in additional populations, using longitudinal study designs, and using higher resolution microarray and locus specific DNA bisulfite sequencing, are required to more fully evaluate this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

DAVID	Database for Annotation, Visualization and Integrated Discovery
FDR	false discovery rate
IP	immunoprecipitated
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAT	model-based analysis of tiling arrays
MeDIP	Methylated DNA Immunoprecipitation
MODY	maturity onset diabetes of the young
ODM	offspring of diabetic mothers
OGTT	oral glucose tolerance test
ONDM	offspring of nondiabetic mothers

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Figure 1.

A. Plot of false discovery rate vs. $-\log(\text{p-value})$ for each possible differentially methylated region. **B.** Relative enrichment in p-value distribution for differential methylation: observed vs. expected under the null hypothesis (based on the random permutations) plotted against $\log(\text{p-value})$. Calculation is restricted to values with $F_{\text{exp}} > 0.02$ to avoid undue influence of extreme values. The maximum enrichment occurred at $p < 0.0077$ ($\text{RE} = 0.103$) and gene regions below this cutoff were considered differentially methylated between ODM and ONDM (4883 gene-regions represented in the boxed area in panel A).

Figure 2.

Pathways for (A) Maturity Onset Diabetes of the Young (hsa04950), (B) type 2 diabetes (hsa04930), and (C) Notch signaling pathway (hsa04330); adapted from Kanehisa, et al [25]. Genes in the pathways which are hypermethylated in ODM compared with ONDM are shown in red boxes, while hypomethylated genes are shown in blue boxes. Genes that did not show differential methylation are in gray boxes. Boxes for *INS* and *CTBP2* are marked with both red and blue indicating that two separate regions with different methylation status overlapped with the promoter of these two genes.

Table 1

Characteristics of subjects from two sample groups

	Offspring of diabetic mothers ODM	Offspring of non-diabetic mothers ONDM	ANOVA P
n	14	14	
Male/Female (n)	7/7	7/7	
Age (mean $\pm$ SD) years	15.5 $\pm$ 5.1	16.2 $\pm$ 5.8	0.4452
BMI (mean $\pm$ SD) kg/m ²	33.4 $\pm$ 7.5	26.0 $\pm$ 8.0	0.0061
HbA1c (mean $\pm$ SD) %	5.4 $\pm$ 0.4	4.9 $\pm$ 0.4	0.0022
Fasting Glucose (mean $\pm$ SD) mmol/l	5.22 $\pm$ 0.50	5.01 $\pm$ 0.37	0.1563
2h Glucose (mean $\pm$ SD) mmol/l	6.17 $\pm$ 1.45	5.72 $\pm$ 1.48	0.4299
Age of Mother at delivery (mean $\pm$ SD)	32.5 $\pm$ 6.2	31.7 $\pm$ 4.9	0.4531
Months between maternal OGTT and delivery (mean $\pm$ SD)	2.4 $\pm$ 1.6	2.5 $\pm$ 1.7	0.8961
Glycemia of mother (before delivery):			
2h post load plasma glucose (mmol/l)	11.1	< 7.8 mmol/l	
Glycemia of mother (1 yr after delivery):			
2h post load plasma glucose (mmol/l)		< 11.1 mmol/l	

Table 2

Gene promoters overlapping with the 20 most differentially methylated regions

Gene Symbol	Transcript ID	Distance to TSS	chr	region start	region end	Probes in region	MAT score	p-value (region)	FDR
USP17	NM_001105662	0	4	8933730	8980813	1248	-5.032	6.17×10^{-6}	0.787
USP17L6P	NR_027279	0	4	8933730	8980813	1248	-5.032	6.17×10^{-6}	0.787
CACNA1B	NM_000718	0	9	139887340	139907599	526	-5.182	6.23×10^{-6}	0.854
MIR3689A	NR_037460	0	9	136879570	136885579	163	-5.642	6.23×10^{-6}	0.854
MIR3689B	NR_037469	0	9	136879570	136885579	163	-5.642	6.23×10^{-6}	0.854
GPHA2	NM_130769	-699	11	64460636	64462943	51	-4.868	6.25×10^{-6}	0.854
TXNDC5	NM_001145549	0	6	7854095	7858636	115	-5.063	6.37×10^{-6}	0.854
PRIM2	NM_000947	0	6	57289267	57295098	148	4.754	1.27×10^{-5}	0.854
HSPG2	NM_005529	281	1	22134306	22136057	46	-4.890	1.27×10^{-5}	0.854
REXO1L1	NM_172239	0	8	86760539	86763506	80	-4.516	2.50×10^{-5}	0.854
REXO1L2P	NR_003594.1	0	8	86760539	86763506	80	-4.516	2.50×10^{-5}	0.854
WNT2	NM_003391	0	7	116739696	116754152	379	4.364	3.08×10^{-5}	0.854
LARP1	NM_015315	-672	5	154070678	154071983	30	4.378	3.08×10^{-5}	0.854
IL18BP	NM_001039659	0	11	71382968	71405653	595	-4.489	3.12×10^{-5}	0.854
EIF2C2	NM_001164623	457	8	141710845	141714372	79	-4.476	3.12×10^{-5}	0.854
CYP21A2	NM_000500	0	6	32105054	32127211	571	4.478	3.18×10^{-5}	0.854
TNXB	NM_032470	0	6	32105054	32127211	571	4.478	3.18×10^{-5}	0.854
TUBGCP5	NM_001102610	0	15	20373876	20386409	333	-4.536	3.70×10^{-5}	0.854
FOXB2	NM_001013735	0	9	78815937	78831253	400	-4.266	3.74×10^{-5}	0.854
SNED1	NM_001080437	0	2	241578984	241592367	344	-4.260	3.74×10^{-5}	0.854

Table 3

KEGG pathways enriched for methylated regions (DAVID Functional Annotation Tool)

Pathway	Description	Count [*]	% [†]	p-value [‡]	Fold Enrichment [§]	Bonferroni	Benjamini	FDR
hsa04950	Maturity onset diabetes of the young	12	0.30	0.0070	2.322	0.749	0.292	8.423
hsa04930	Type II diabetes mellitus	18	0.44	0.0099	1.853	0.857	0.277	11.663
hsa04330	Notch signaling pathway	17	0.42	0.0223	1.750	0.988	0.468	24.559
hsa04912	GnRH signaling pathway	32	0.79	0.0066	1.580	0.726	0.350	7.920
hsa03320	PPAR signaling pathway	22	0.54	0.0348	1.542	0.999	0.500	35.750
hsa04370	VEGF signaling pathway	23	0.57	0.0461	1.484	1.000	0.509	44.565
hsa04512	ECM-receptor interaction	25	0.62	0.0450	1.457	1.000	0.528	43.714
hsa04310	Wnt signaling pathway	44	1.09	0.0098	1.429	0.855	0.320	11.573
hsa04360	Axon guidance	36	0.89	0.0363	1.371	0.999	0.483	36.995
hsa04010	MAPK signaling pathway	74	1.83	0.0035	1.351	0.501	0.293	4.330
hsa05200	Pathways in cancer	90	2.22	0.0013	1.348	0.227	0.227	1.629
hsa04510	Focal adhesion	53	1.31	0.0312	1.295	0.998	0.498	32.678
hsa04080	Neuroactive ligand-receptor interaction	65	1.61	0.0307	1.258	0.998	0.535	32.298

^{*} Number of input genes that are involved in a pathway

[†] Percentage of user's input genes involved in a pathway

[‡] EASE Score, a modified Fisher exact p-value, for gene-enrichment analysis.

[§] Ratio of percentage of user's input genes involved in a pathway to the percentage of pathway genes in the background list

^{||} Multiple testing correction techniques based on Bonferroni, Benjamini-Hochberg, and FDR (False Discovery Rate) to globally correct enrichment p-values to control family-wise false discovery rate under certain rate.