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Epigenetic Signature of Impaired Fasting Glucose in the Old Order Amish

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Abstract

Introduction—Type 2 Diabetes (T2D) is a common chronic disease with substantial disease burden and economic impact. Lifestyle changes can significantly alter the course of the disease, if detected at an early stage. DNA methylation signature may serve as a biomarker for early detection of increased T2D risk.

Design—DNA methylation profiling was performed using the Illumina Infinium Human Methylation 450K Bead chip array in 24 normoglycemic Old Order Amish (OOA) individuals who later developed Impaired Fasting Glucose (IFG) (cases), and 24 OOA individuals who remained normoglycemic after an average follow up of 10 years (controls). Cases and controls were matched on age, sex, BMI, baseline fasting glucose, and glucose level after 2 h from 75 g Oral Glucose Tolerance Test (OGTT).

Results—Association analysis found no significant difference in either global methylation or individual probe methylation between cases and controls, however, the top 34 suggestive significant sites were located in genes with interesting biological links to T2D and glycemic traits. These genes include *BTC* that plays a role in pancreatic cell proliferation and insulin secretion, *ITGA1* a known bone mineral density gene that was recently found to be associated also with T2D and glycemic traits, and may explain the link between T2D and BMD, and *RPTOR* and *TSC2* both of which are part of insulin signaling pathway.

Conclusions—These results may shed light on the initiation and development of hyperglycemia and T2D and help to identify high risk individuals for early intervention; however, further studies are required for validation.

Keywords

Old Order Amish (OOA); DNA methylation; Impaired Fasting Glucose (IFG)

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Conflicts of Interest

Alan R Shudiner is an employee of Regeneron Pharmaceutical Inc. Jeffrey R. O'Connell is a consultant for Regeneron Pharmaceuticals, Inc.

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Introduction

Type 2 Diabetes (T2D) is a common chronic disease with substantial disease burden and economic impact. Lifestyle changes can significantly alter the course of the disease, notably if detected prior to having established diabetes. However, needed pre-clinical markers of disease are still lacking to enable early detection. Such markers would potentially have the greatest impact on disease progression by allowing targeted early interventions. Besides genetics and environmental factors, epigenetic changes have emerged as playing a significant role in T2D development and progression. Indeed, DNA methylation, a key epigenetic modification of gene expression, has been linked to several diseases and traits [1,2], including T2D [3–8] and its glycemic traits [9,10]. An early Epigenome-Wide Association Study (EWAS) found significant hypomethylation in the *FTO* gene within T2D patients compared to healthy controls [11]. Using pancreatic islets from 15 T2D patients and 34 controls, significant differential methylation levels were identified in several genes including some well-known diabetes related genes like *TCF7L2* and *KCNQ1* [4]. Subsequent studies identified several loci but results did not overlap [3,8–9] with the exception of *TXNIP* which has been found to be significantly associated with T2D in 3 recent studies [5–7].

These results suggest that the DNA methylation signatures may serve as biomarkers for early detection of increased T2D risk. However, these studies were cross sectional, and thus unable to distinguish between changes in methylation that predate the development of diabetes versus those caused by it. The cross sectional nature of these studies may, at least partially, explain the lack of consistency between their results.

To overcome this limitation we used a longitudinal study design to identify differentially methylated sites between 24 normoglycemic Old Order Amish (OOA) individuals who later developed IFG (cases), and 24 OOA individuals who remained normoglycemic after an average follow up of 10 years (controls).

Dissecting epigenetic causes and consequences of the disease can help in early disease prediction, better understanding of the molecular mechanisms of the disease, and identifying potential new drug targets.

Materials and Methods

Study samples

The OOA first emigrated from Central Europe (mainly Switzerland) to America in the 1700s. Currently, there are about 30,000 OOA living in Lancaster County. They maintain detailed genealogical records and nearly all Amish can trace their ancestry back to approximately 750 original founding individuals. The Lancaster Amish are mostly farmers, do not use modern technology, and have a very homogeneous life style. Both the environmental and genetic homogeneity of this community make it excellent for genetic and epigenetic studies, as methylation levels can be affected by both genetic and environmental factors. The Amish Complex Disease Genetic Program [12] has been studying a variety of complex traits and diseases over the last 20 years and has recruited more than 7,000 subjects,

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many of whom have participated in several studies [13–16]. By searching our database, we were able to identify 24 normoglycemic OOA individuals (70 ≤ fasting glucose (FG) <100 mg/dL and 75 g Oral Glucose Tolerance Test (OGTT) post-load glucose: <140 mg/dL) who later developed IFG (100 ≤ FG < 126 mg/dL) (cases), and 24 OOA individuals who remained normoglycemic after an average follow up of 10 years (controls). Cases and controls were matched on age, sex, BMI, baseline FG, and glucose levels 2 h post 75 g OGTT.

The samples used in this study were selected from the participants of Amish Family Diabetes Study [13]. The study protocol was approved by the institutional review board at the University of Maryland. Informed consent was obtained from each of the study participants.

Methylation chip and QC

Genome-wide DNA methylation profiling for the baseline whole blood was performed using the Illumina Infinium Human Methylation 450K Bead chip array (450K) [17]. Sample preparation and hybridization were performed by the same technician, and the arrays were processed using the same scanner to reduce batch effects. Genome Studio (GS) (V2011.1) was used to process the raw intensity files and perform both sample and probe QC. GS normalization and background correction were applied according to Illumina's recommendation. Examining internal control probes did not identify any samples for removal, and all samples had >95% of probes detected with average p-value <0.01. Probes with average detection p-value >0.01 or call rate <95% were removed from analysis. In addition sex chromosome probes and previously identified cross-reactive probes [18–20] were excluded, thus leaving 318,165 probes for analysis, of which only 274,088 were successfully tested (the model didn't converge for some probes with low variation). Blood cell subtype composition was estimated using the Housman method [21,22].

Statistical analysis

To test global methylation, the mean beta value of all probes per individual was calculated. To test for methylation per different genomic annotation, we also calculated the mean beta values for probes within each genomic region or distance to CpG Island according to Illumina's manifest.

Mixed model analysis was used to calculate the inverse normalized residual of the methylation level of each probe (in single probe analysis or mean values in global/regional methylation test) adjusted for age, sex, cell subtype composition, and technical covariates as fixed effects, as well as the family structure as a random effect. Linear regression was used to test the association between each inverse normalized residual as a dependent variable with case control status. A conservative Bonferroni corrected p value of 1.8E-7 was used as the significance threshold.

Results

The overall design of this study is shown in Figure 1. The study used 24 normoglycemic OOA individuals (70 ≤ FG < 100 mg/dL and 75 g OGTT post-load glucose: <140 mg/dL)

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who later developed Impaired Fasting Glucose (IFG) (100 ≤ FG < 126 mg/dL) (cases), and 24 OOA individuals who remained normoglycemic after an average follow up of 10 years (controls). Cases and controls were matched on age, sex; BMI, baseline fasting glucose, and glucose level 2 h post 75 g OGTT as shown in Table 1.

There was no significant difference in global methylation between cases and controls (p=0.22). There was also no difference in methylation levels for probes annotated based on their gene region or distance to CpG Island (Figures 2 and 3) between cases and controls. However, the pattern of methylation across the genome showed the lowest mean and variance methylation level in the promoter region that includes the 1st exon, TSS200 (200 base pairs within the Transcription Start Site), 5' UTR and TSS1500 (1500 base pairs within the TSS excluding the TSS200 region) while higher methylation level and variance was found in the gene body, 3' UTR, and intergenic probes. In terms of distance from islands, the lowest mean and variance methylation level was found for probes located in CpG islands, followed by those in shores (0–2 kb around the CpG Island). Probes in shelves (2–4 kb around the CpG Island) and open sea had the highest mean and variance.

Of the 274,088 successfully tested probes, we identified 17,702 probes with p-value <0.05 which is significantly higher than that expected by chance alone (p=2.2E-16, X^2 test). While no probe reached the genome wide significance threshold of 1.8E-7, we identified 34 probes with suggestive p-values <1.0E-5, 23 of which are located inside genes (Table 2). Nine of the 34 sites were hypomethylated in cases, while 25 were hypermethylated. Twelve of the 34 sites were located inside enhancer region.

None of the top 34 probes was previously reported among 1828 probes that were identified in previous studies [3–7], however, out of these 1828 probes, 1219 were tested in our study and 80 of them had a p-value <0.05, including those in insulin like growth factor 1 receptor (*IGF1R*).

Discussion and Conclusion

The aim of this study was to identify methylation sites associated with IFG that could be used as biomarkers for early detection of individuals at high risk of T2D to target for preventive intervention. We compared methylation profiles of 24 normoglycemic Old Order Amish (OOA) individuals who later developed IFG (cases), and 24 OOA individuals who remained normoglycemic after an average follow up of 10 years (controls).

We were unable to find significant differences in global methylation levels by gene region or distance to island between cases and controls in our study. To date the evidence for the association between global methylation and T2D or glycemic traits has been inconsistent, ranging from positive association to negative association and no association at all [23]. The differences in study design, power, tissue, and methylation-profiling technique may explain these discrepancies.

This distribution of the mean and variance of the methylation levels based on genomic region and distance to CpG islands in our blood samples was similar to the pattern reported for adipose tissue [3,24] and human pancreatic islets, alpha and beta cells [4], adding

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support to the use of blood samples in T2D studies as blood reflects the same methylation pattern of other T2D relevant tissues. Promoter regions are conserved and have low variation in methylation due to their important role in gene regulation [24]. Highly variable hypermethylated sites in intergenic and open sea regions were found to be enriched with significantly differentially methylated sites between T2D cases and controls [4], showing that highly variable sites are more likely to be associated with T2D and glycemic traits.

While our single probe analysis did not identify any significant association with case-control status after multiple testing, our 34 top identified probes included several within genes with interesting biological links to T2D and glycemic traits. Identified genes include the Betacellulin gene *(BTC)* that plays a role in pancreatic cell proliferation and insulin secretion and the integrin subunit alpha 1 *(ITGA1)*, a known bone mineral density (BMD) gene that was recently found to be associated also with T2D and glycemic traits, providing a possible explanation of the link between T2D and BMD [25], as well as the regulatory associated protein of MTOR complex 1 gene *(RPTOR)* and the Tuberous Sclerosis 2 gene *(TSC2)* both of which are part of insulin signaling pathway [25].

The major strength of our study is its unique design, however, this unique design made it hard to find a large number of samples that satisfy the inclusion criteria. Among our 7,313 banked Amish samples, 1,442 of them have repeated measures, but the only samples identified to fit the study design criteria were these 48. All other repeated samples were either normoglycemic at all-time points or diabetic/prediabetic at all-time points, illustrating the challenges most studies will have to achieve well-powered sample sizes. The ideal study design to distinguish between methylation sites that cause disease or a consequence of disease is a longitudinal study that follows up a very large number of individuals over several decades, but this is very costly and lengthy endeavor. These results may shed light on the initiation and development of hyperglycemia and T2D and help to identify high-risk individuals for early intervention; however, further studies are required for validation.

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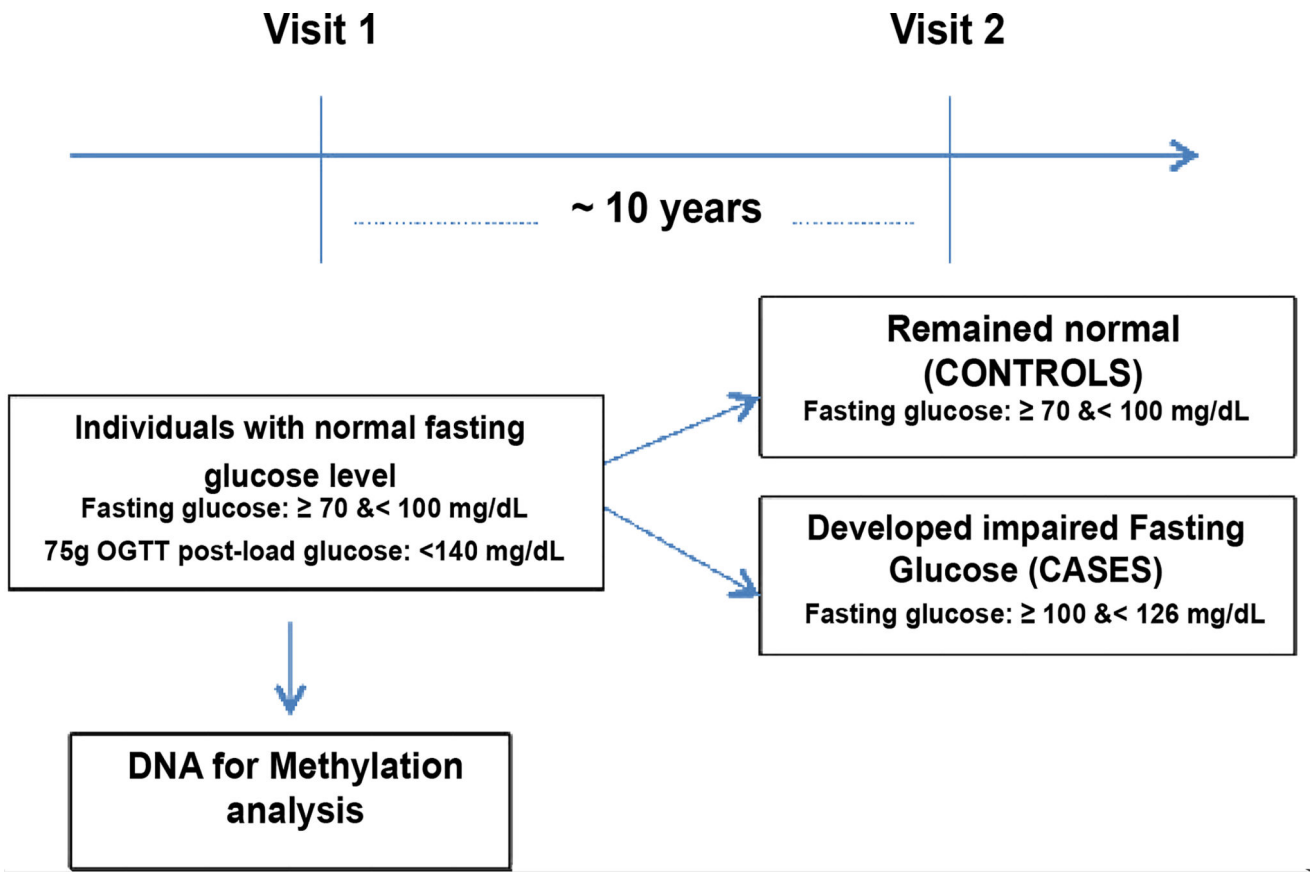

Figure 1.
Flow chart of the study design.

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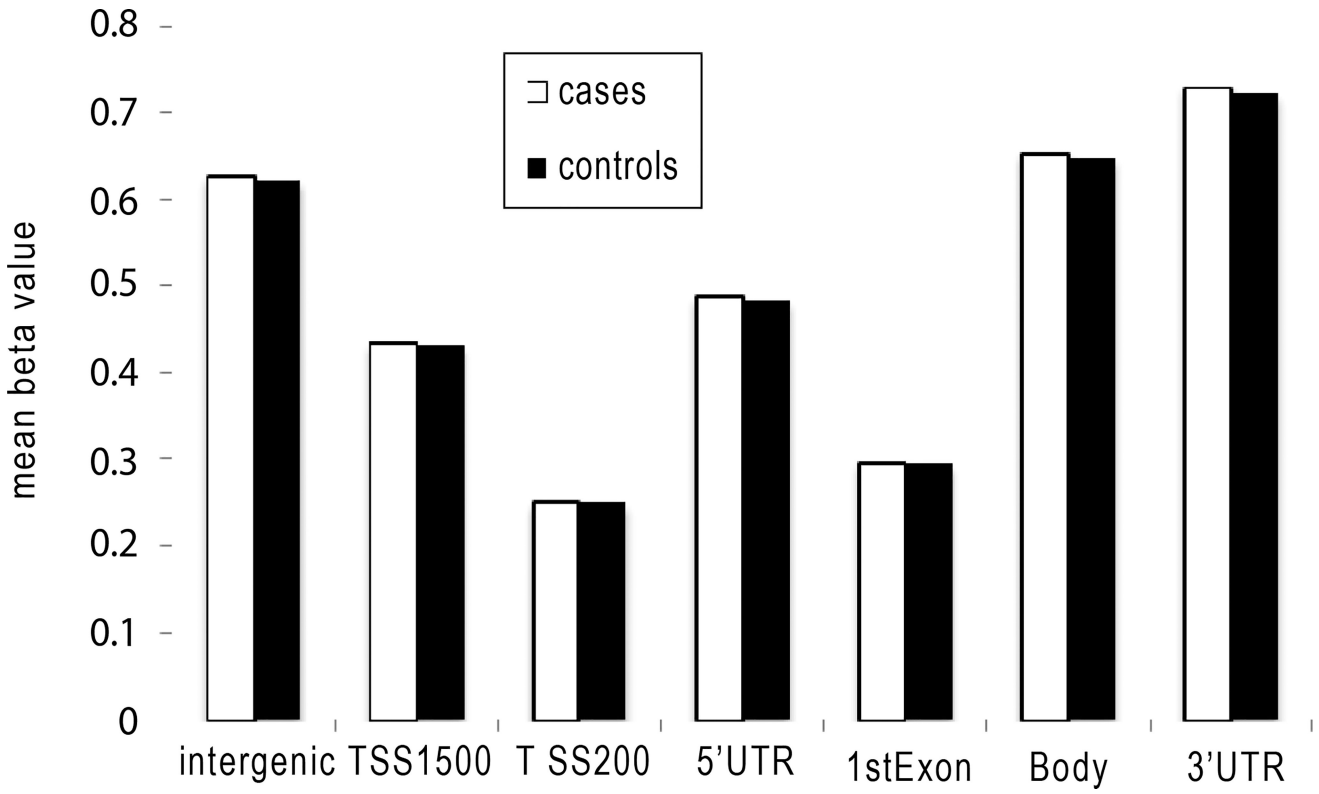

Figure 2.
The methylation pattern in blood samples by gene region.

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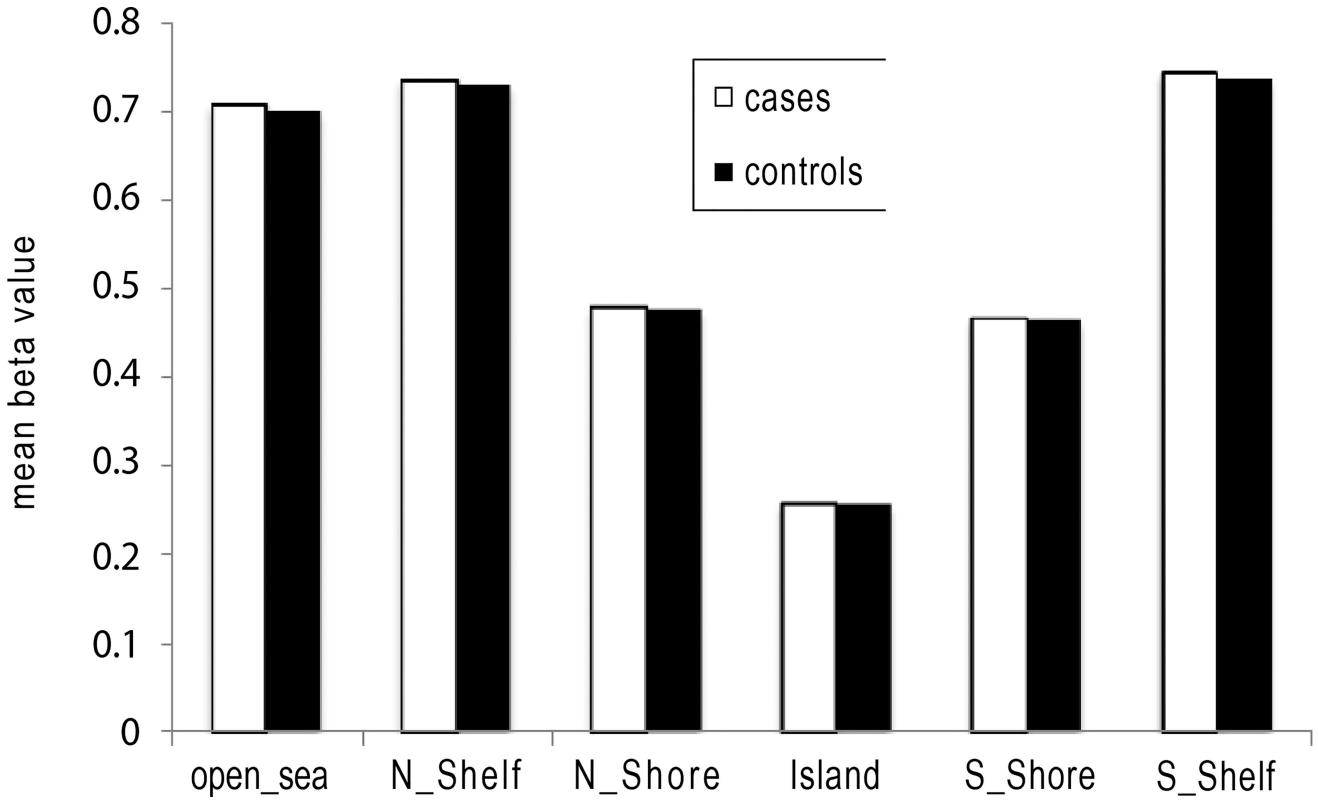

Figure 3.
The methylation pattern in blood samples by CpG island region.

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Table 1

Descriptive characteristics of the study population in mean (sd).

	Cases (n=24)	Controls (n=24)	p-value
Age at baseline, years	42.5 (6.9)	42.4 (7.7)	0.9
BMI at baseline, kg/m^2	29.3 (3.5)	28.4 (3.6)	0.3
Fasting glucose at baseline, mg/dL	91.1 (5.2)	88.7 (5.2)	0.1
2h OGTT at baseline, mg/dL	103.6 (18.0)	102.0 (22.2)	0.7
Duration of follow-up, years	9.5 (3.4)	10.2 (3.7)	0.5
Fasting glucose at follow-up, mg/dL	106.6 (6.1)	85.9 (7.3)	6.93E-14

10 male pairs, 14 female pairs, No medication

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Table 2

The top 34 probes associated with impaired fasting glucose.

ILMNID	Hypo/Hyper	p-value	Chr	Pos	Gene	Gene region	Relation to island	Enhancer
cg02333875	↑	7.19E-06	6	29618205			S_Shore	TRUE
cg23606751	↓	1.67E-05	14	62228873	SNAPC1	TSS1500	N_Shore	
cg07875069	↑	2.27E-05	7	129985025	CPA5	1stExon		TRUE
cg19412743	↓	2.95E-05	4	190747919				TRUE
cg13916874	↑	3.14E-05	6	4286300			S_Shelf	
cg01403103	↓	3.33E-05	16	112852	RHBDF1	Body	Island	
cg17257444	↑	3.76E-05	11	59224044	0R4D6	TSS1500		
cg22387253	↑	3.95E-05	15	61310104	RORA	Body		TRUE
cg21464903	↑	4.12E-05	8	41220059				TRUE
cg14442421	↑	4.31E-05	9	19788257	SLC24A2	TSS1500	Island	
cg21062902	↑	4.34E-05	6	34162022			N Shelf	
cg22212260	↑	4.62E-05	3	33468135	UBP1	Body		TRUE
cg16218910	↑	4.76E-05	17	78878579	RPTOR	Body	N_Shore	
cg10796022	↑	4.83E-05	8	61227705				TRUE
cg23254538	↑	4.90E-05	6	31556919	NCR3	3'UTR		
cg10981651	↑	4.98E-05	5	153853545			Island	TRUE
cg08031810	↑	5.10E-05	2	52856918			Island	
cg23200357	↑	5.16E-05	21	33108677			S_Shelf	
cg19872284	↑	5.23E-05	10	49607582				TRUE
cg21944022	↑	5.28E-05	5	178288650	ZNF354B	Body	S_Shore	
cg13491945	↑	5.37E-05	12	107078410	RFX4	TSS200		
cg11617821	↑	6.17E-05	4	75672135	BTC	3'UTR		
cg06002516	↑	6.32E-05	12	57666521	R3HDIv12	Body		TRUE
cg13853141	↓	6.65E-05	2	98329942	ZAP70	TSS200		
cg25375293	↓	6.87E-05	12	54718868	COPZ1	TSS200		
cg13993764	↑	6.88E-05	4	102269779	PPP3CA	TSS1500	S_Shore	

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ILMNID	Hypo/Hyper	p-value	Chr	Pos	Gene	Gene region	Relation to island	Enhancer
cg07904475	↓	7.04E-05	4	151500931	LRBA	Body	Island	TRUE
cg21120478	↓	7.78E-05	12	6833063	COPS7A	TSS200	Island	
cg01382688	↓	7.91E-05	2	131672054			N_Shore	
cg00154957	↑	8.84E-05	1	109850562	IvlYBPHL	TSS1500		
cg10196026	↑	9.01E-05	5	35003575	AGA¯2	Body		
cg13050884	↑	9.45E-05	3	171976122	FNDC3B	Body		TRUE
cg16997488	↓	9.70E-05	5	52083949	ITGA1:PELO	1stExon	Island	
cg00096979	↑	9.93E-05	16	2116141	TSC2	Body	S_Shore	

ILMNID: Illumina probe ID, ↑ Hypermethylated in cases, ↓ Hypomethylated in cases, Chr: Chromosome, Pos: Position build 37