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DNA methylation near the *INS* gene is associated with *INS* genetic variation (rs689) and type 1 diabetes in the Diabetes Autoimmunity Study in the Young

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Abstract

Objective—Mechanisms underlying the role of non-human leukocyte antigen (HLA) genetic risk variants in type 1 diabetes (T1D) are poorly understood. We aimed to test the association between methylation and non-HLA genetic risk.

Methods—We conducted a methylation quantitative trait loci (mQTL) analysis in a nested case-control study from the Dietary Autoimmunity Study in the Young. Controls (n = 83) were frequency-matched to T1D cases (n = 83) based on age, race/ethnicity, and sample availability. We evaluated 13 non-HLA genetic markers known to be associated with T1D. Genome-wide methylation profiling was performed on peripheral blood samples collected prior to T1D using the Illumina 450 K (discovery set) and Infinium methylation EPIC beadchip (EPIC validation) platforms. Linear regression models, adjusting for age and sex, were used to test for each single nucleotide polymorphism (SNP) -probe combination. Logistic regression models were used to test the association between T1D and methylation levels among probes with a significant mQTL. A meta-analysis was used to combine odds ratios from the two platforms.

Results—We identified 10 SNP-methylation probe pairs (false discovery rate (FDR) adjusted $P < .05$ and validation $P < .05$). Probes were associated with the *GSDMB*, *C1QTNF6*, *IL27*, and *INS* genes. The cg03366382 (OR: 1.9, meta- $P = .0495$), cg21574853 (OR: 2.5, meta- $P = .0232$), and cg25336198 (odds ratio: 6.6, meta- $P = .0081$) probes were significantly associated with T1D. The three probes were located upstream from the *INS* transcription start site.

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AUTHOR CONTRIBUTIONS

P.C. designed the study, performed the data analysis, interpreted the data, and drafted the manuscript. J.N., K.K., and L.V. designed the study, interpreted the data, and edited the manuscript. A.S., M.R., B.F., F.D., and I.Y. reviewed and edited the manuscript.

Peer Review

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Conclusions—We confirmed an association between DNA methylation and rs689 that has been identified in related studies. Measurements in our study preceded the onset of T1D suggesting methylation may have a role in the relationship between *INS* variation and T1D development.

Keywords

INS; islet autoimmunity; methylation; mQTL; T1D

1 | INTRODUCTION

Type 1 diabetes is a complex autoimmune disease with a strong, underlying genetic component.¹ The majority of genetic effects map to the human leukocyte antigen (HLA) region of chromosome 6, a region that plays a key role in immune system function.^{1–3} Extensive work has been performed to understand the complex relationship between HLA variation and changes in immune cell receptor structure and function.^{4,5} The remaining genetic variation maps to non-HLA regions. The insulin gene (IDDM2 locus) is regarded as the second most important gene locus in type 1 diabetes (T1D).⁶ Risk at the IDDM2 locus is due to a haplotype consisting of a variable number tandem repeat⁷ and genetic variation at the rs689 SNP, both located upstream from the *INS* gene transcription start site (TSS).⁸ The two sources of genetic variation exist in perfect linkage equilibrium.⁸

The mechanistic relationship between the non-HLA genetic variants and T1D is not well understood. Furthermore, increasing incidence of T1D points to an important role for environmental factors in disease pathogenesis. Epigenetic mechanisms have been proposed as a potential mediator of the link between genes, environment, and disease onset.^{4,9,10} DNA methylation is a frequently studied epigenetic modification that is capable of influencing gene expression.¹¹ Previous studies in T1D have identified associations between methylation and T1D.^{12–17} Of note, analysis in human pancreatic islets also identified a correlation between methylation and mRNA expression as well as insulin secretion, providing evidence of a role of methylation in pancreatic islet function.¹⁵ Together, this evidence highlights the role of methylation as a potential biomarker of the interplay between genetic risk, environmental exposure, and T1D risk.

Targeted and untargeted studies have identified associations between DNA methylation and genetic variation at loci relevant to the T1D disease process^{13,18,19} as well as associations between DNA methylation and T1D.^{13,16,17,20} However, many studies are limited in that methylation measurements were obtained after disease onset, raising concerns that methylation differences may have occurred in response to the disease process and not due to genetic regulation. Previous studies have also not explicitly tested the interrelationships between DNA methylation, measured prior to T1D, genetic variation, and the onset of T1D in a dataset that includes all three of these critical measurements. The purpose of this study was to build on previous genetic and epigenetic association studies by testing the association between non-HLA SNPs and methylation measurements obtained prior to the development of T1D. We performed methylation quantitative trait loci (mQTL) analyses in an existing nested case-control study from the Diabetes Autoimmunity Study in the Young (DAISY).

2 | RESEARCH DESIGN AND METHODS

2.1 | Participants

DAISY is a large cohort study that is prospectively following children identified at birth as having one or more of the HLA-DR or DQ high risk gene alleles ($n = 1408$) as well as siblings or offspring of individuals with T1D ($n = 1139$). Demographic data and blood samples are obtained from individuals at regular intervals. The design of the study has been described in greater detail in previous publications.^{21,22} Informed consent and/or assent, as appropriate, is obtained from all individuals enrolled in the DAISY study. The DAISY study as well as secondary analyses of existing data were reviewed and approved by our institutional review board.

2.2 | Data collection

A nested case-control study from DAISY was used in the current study. Controls were selected from the pool of subjects in DAISY who never developed islet autoimmunity (IA) and/or T1D. Controls were frequency matched to T1D cases based on age, race/ethnicity, and sample availability. IA was defined as the presence of autoantibodies against one or more of the following antigens on two or more consecutive study visits: insulin, glutamic acid decarboxylase autoantibody, zinc transporter 8, or protein tyrosine phosphatase autoantibody. Clinical T1D was defined according to the ADA criteria.²³ Individuals were split into a discovery set and a validation set for the mQTL portion of the analysis. Due to the large number of comparisons made in the mQTL portion of the analysis, we used a conservative, validation-based approach to confirm associations between methylation and genotype. All available samples were used in the statistical analysis.

2.3 | Methylation measurements

Peripheral blood samples are prospectively collected from individuals enrolled in DAISY. Among T1D cases, we used the first blood sample collected after the onset of IA, that is, the first available sample among two or more consecutive visits in which autoantibodies were detected. Among controls, we used an age matched blood sample. Genome wide methylation profiling was performed in the blood samples using the Infinium HumanMethylation 450 K Beadchip platform (Illumina, San Diego, California) or the Infinium HumanMethylation EPIC Beadchip platform. The 450 K platform was considered the discovery set whereas the EPIC platform was considered the validation set. Subjects were randomly assigned to the replication or the discovery set. The minfi (v1.12.0) package²⁴ in R (v3.5.2) was used to perform quality control (QC) checks at the sample level. Samples that failed any of the standard QC checks were treated as missing data. Non-autosomal CpGs were excluded. Data from both platforms were normalized and had probe-level QC performed using the SeSAMe²⁵ package (v1.0.0) in R (v3.5.2). The normalized M -values were used in all statistical analyses. Beta values, representing percent methylation, were used to facilitate biological interpretation.

2.4 | Genotyping

Individuals enrolled in DAISY were genotyped for selected non-HLA SNPs. Genotyping included the following methods: custom Taqman SNP genotype-based OpenArray platform (Applied Biosystems, Carlsbad, California, rs229541, rs2292239, rs7020673, rs2290400, rs4788084, rs11203203, rs3788013, and rs10517086), linear array or immobilized probe methods (as described by Mirel et al,²⁶ INS23Hph1/rs689, rs2476601), and Illumina GoldenGate Beadexpress assays (rs12251307, rs11755527, and rs10509540). The methodology for the SNP collection, processing, and analysis in DAISY has been described previously.^{27–33}

SNPs were selected for consideration in the current study if they were significantly (nominal $P < .05$) related to onset of T1D in previous DAISY publications and/or were included in the T1D genetic risk score model proposed by Winkler et al³⁴ that was applied to the DAISY study population in Frohnert et al.³⁰ SNPs selected for the analysis include the following: rs2476601 (*PTPN22*), rs689 (*INS*), rs11203203 (*UBASH3A*), rs3788013 (*UBASH3A*), rs229541 (*CIQTNF6*), rs10517086 (unknown), rs7020673 (*GLIS3*), rs12251307 (*IL2RA*), rs2292239 (*ERBB3*), rs2290400 (*GSDMB*), rs11755527 (*BACH2*), rs4788084 (*IL27*), and rs10509540 (*RNLS/C10orf59*) (Table S1).

2.5 | Statistical Analysis

We used a mQTL analysis to identify methylation sites that are associated with the non-HLA SNPs. The 450 K platform set was used in the discovery analysis. Multiple linear regression analyses were used to test the association between all SNPs and methylation at all probes (ie, SNP probe pairs). Age and sex were included in the models as precision variables. Significance was assessed based on the Benjamini–Hochberg³⁵ FDR-corrected level of 0.05. This analysis identified methylation and SNP pairs acting in *cis* or *trans*. *Cis* pairs were defined as all SNP CpG pairs within one million bases.³⁶

SNP probe pairs that were significant at the FDR adjusted P value of .05 were tested in the validation EPIC set. SNP probe pairs that were significant at an alpha level of 0.05 in the validation were considered for T1D methylation association testing.

We also tested whether probes significantly related to SNPs in the mQTL analysis were related to T1D using a differentially methylation position (DMP) analysis. Probes located near a known SNP were excluded from analysis because it is not possible to determine whether the measurement signal reflects methylation or whether it reflects genetic variation.³⁷ Multiple logistic regression analyses were used to test the association between probes and T1D. Age was adjusted for in the models. A fixed-effects meta-analytic framework was used to combine probe-disease associations from the 450 K and EPIC platforms. Pooled odds ratios were obtained using the metafor R (V 2.1–0) package.³⁸

2.6 | Exploratory Analyses

Methylation data were also obtained prior to the onset of IA for a subset of T1D cases ($n = 40$) and controls ($n = 42$). We used these additional data to explore whether differences between cases and controls observed after the onset of IA were also present prior to IA

onset. Descriptive statistics were used to summarize the distribution of the beta values in the two groups prior to the onset of IA.

3 | RESULTS

The distribution of age, sex, first-degree relatives with T1D, and ethnicity was similar between T1D cases and controls in both the validation and discovery sets. The high risk DR3/4 genotype was more prevalent in T1D cases compared to controls (Table 1).

3.1 | SNP probe pairs

The mQTL analysis was used to test the association between $n = 454\,078$ methylation sites located across the genome and the 13 SNPs outlined in Table S1. We identified 13 significant SNP-methylation probe pairs ($n = 4$ unique SNPs and $n = 13$ unique methylation probes, Table 2 and Figure S1). All significant mQTLs were *cis*-regulated and located on chromosomes 11, 16, 17, and 22 (Table 2). The methylation sites were most frequently open sea probes (69%) followed by shore (15%), island (8%), and shelf (8%) probes. In the validation set, 10 of the 13 probes identified were confirmed. Among the three probes that were not confirmed, two were not available on the EPIC platform. The remaining probe, cg18711369, was not significantly associated with rs2290400 in the validation analysis (Table 2).

Based on functional annotation data on the 13 probes from the University of California Santa Cruz genome browser, 8% are located in a gene body, 54% are located near the TSS of a gene, 8% are located near the 3'UTR region, 15% are located in the 5'UTR region, 8% are located within the first exon, and 23% map to a region that associates with known regulatory functions (Table S2).

3.2 | Differentially methylated positions

We tested whether probes identified in the mQTL and confirmed in the validation mQTL were significantly different between T1D cases and controls. Based on the meta-analysis, we identified three probes that were significantly differentially methylated between cases and controls. Methylation levels at all three probes were increased in cases compared to controls (Table 3 and Figure 1). All three probes were located near the *INS* gene on chromosome 11.

It is possible that methylation levels could also be influenced by the ongoing autoimmune process. Therefore, we explored whether methylation levels observed after onset of IA were also present prior to the onset of IA. For the cg03366382 probe, median methylation levels were 91.5% (range: 78.8%–94.0%) in cases compared 89.9% (range: 78.2%–93.7%) in controls. For the cg25336198 probe, median methylation levels were 64.3% (range: 55.8%–73.2%) in cases compared 62.8% (range: 54.0%–72.6%) in controls. For the cg21574853 probe, median methylation levels were 84.6% (range: 70.1%–90.8%) in cases compared 82.6% (range: 69.9%–89.7%) in controls (Figure 2).

4 | DISCUSSION

The role of genetics in T1D is well established. However, our understanding of the mechanistic connection between known genetic variants, especially non-coding genetic variation, and disease is limited. We explored the association between causative non-HLA genetic variants and DNA methylation before T1D diagnosis. We identified 10 SNP probe pairs significantly related to methylation levels prior to the development of T1D.

All but one of the SNP probes identified in the mQTL was associated with methylation in *cis*, meaning the CpG sites were within one million base pairs of the SNP. Cis-mQTLs represent a distinct type of epigenetic effect that should be interpreted accordingly.³⁹ In contrast to traditional epigenome-wide association studies that aim to identify epigenetic changes attributable to environmental effects, mQTL analyses identify genetic-epigenetic interactions that should be interpreted as a type of genetic effect. Although mQTLs are distinct from environmental mediated effects, mQTLs have biologically relevant implications for disease based on their role in transcription factor binding, gene expression, chromatin accessibility, and chromatin looping.³⁹ In the context of complex diseases, *cis*-mediated allelespecific methylation represents a potential mechanism for the association between non-coding genetic variation and disease.⁴⁰

The SNP methylation hits in the mQTL portion of our analysis support the work of Kindt et al,¹⁸ as they also found the INS 5' variable tandem repeat (in perfect LD with rs689 SNP) AA genotype was associated with hypermethylation relative to the higher risk A/T and T/T genotypes. Our study also replicated a significant probe SNP pair from Kindt et al¹⁸ that maps to the *CIQTNF6* gene (rs229541 and cg05209515). Ye et al¹⁹ also tested the association between 55 non-HLA SNPs and DNA methylation. Based on a two-stage Mendelian randomization design, Ye et al¹⁹ identified five loci, *ITGB3*, *AFF3*, *PTPN2*, *CTSH*, and *CTLA4* where the association between genetic variation and disease is potentially mediated by methylation levels.

A potential limitation of related studies^{13,18,19} is that the association between methylation and T1D has not been explicitly tested among a set of T1D cases and controls with both genetic and preonset of T1D methylation data. The nested case-control study design in our study allowed us to test the association between early methylation and later development of T1D. Among the eight candidate probes tested in the DMP, three probes (cg03366382, cg21574853, and cg25336198) were significantly hypermethylated in cases compared to controls. We also explored methylation levels prior to the onset of IA. Methylation patterns before IA were consistent with methylation patterns after IA (Figure 2). Together, these observations are important as the measurements were obtained early in the disease process and, thus, were not due to the autoimmune process, onset of disease (hyperglycemia), and/or management of T1D.

The probes significantly associated with T1D mapped back to the same SNP, rs689, on chromosome 11, located near the *INS* gene, which is regarded as the second most important gene locus in T1D.⁶ An increase in the number of minor alleles at the rs689 SNP is strongly inversely associated with T1D, with an odds ratio of 0.42 ($P < 10^{-100}$).⁴¹ It has been

hypothesized that the minor allele promotes alternative splicing favoring an alternative, shorter isoform that is associated with improved transcriptional efficiency and decreased susceptibility to T1D.⁴² However, Marchand and Polychranokos failed to identify an association between alternative transcript and the presence of the minor allele in pancreatic cells.⁴³

Methylation near the *INS* gene represents an alternative mechanism for the association between the IDDM2 locus and T1D. In the non-obese diabetic mouse model, methylation levels are known to regulate the expression of the *ins1* and *ins2* genes. More importantly, methylation of the insulin gene in beta cells was observed to increase in response to cytokine levels during disease progression, indicating a possible link between immune reactivity, increased methylation, and decreased expression.⁴⁴ In the current study, the direction of association between methylation and T1D was consistent among pre- and post-IA samples (Figures 1(B), (D), and (F), and 2 (A), (B), and (C)), reinforcing the interpretation of mQTLs as inherited epigenetic patterns. Known mechanisms by which mQTLs influence in disease onset, especially alterations in transcription factor binding, should be explored as potential mechanisms that underlie the association between *INS* methylation and T1D.

Two additional case-control studies in human populations^{13,20} have also observed associations between methylation near the *INS* gene and T1D. Fradin et al¹³ reported differential methylation between T1D cases and controls at methylation sites near the *INS* TSS. Four of the sites were hypomethylated whereas one site was hypermethylated in T1D cases compared to controls. Consistent with the results of our study, the rs689 SNP was significantly associated with methylation at four of the six sites studied (see Table S3). Our results at the cg21574853 probe closely mirror those of Fradin et al¹³ at CpG-180 in both disease methylation association, hypermethylation in cases compared to controls, and the rs698 genotype methylation association, increased methylation at the minor, A, allele. In both studies (Table S3), cases were associated with both hyper and hypomethylation relative to controls suggesting the underlying mechanistic relationship between methylation and T1D may be site specific, even within relatively narrow areas of the genome. Using a twin design, Stefan et al²⁰ reported differential methylation between twins discordant for T1D at a probe, cg13993218, near the TSS for the *INS* gene.

There are some limitations of the current study. Each SNP probe pair was evaluated separately. Compensatory effects from other biological mechanisms may complicate the analysis if methylation is strongly associated with a SNP but weakly associated with the disease due to compensation by another source of genetic variation that was not considered. The cross-validation approach was used to minimize false positives during the mQTL portion of the analysis. The subjects included on the discovery and validation platforms were drawn from the same study population, DAISY, and thus the validation analysis does not represent a true replication analysis. DNA methylation measurements were obtained from whole blood samples. As a result, we were not able to report SNP methylation or methylation T1D associations within strata defined by cell subpopulations. Results should be interpreted as an unweighted average of the effects within each cell subpopulation. Finally, samples were obtained during an existing cohort study by an experienced but large study team. Laboratory technicians obtaining and processing samples may differ across the

samples. However, because samples were all obtained prior to onset of disease, we believe the potential bias due to heterogeneity in lab practices was likely to be non-differential.

DNA methylation levels are known to be tissue specific.⁴⁵ The relevance of the mQTL hits identified in whole blood samples in our study to methylation genetic associations in pancreatic cells is not clear. Olsson et al¹⁵ performed an mQTL analysis in pancreatic cells that highlights the association between methylation and genetic variation in pancreas cells and also provides an opportunity to review overlap between mQTL hits in our study and this pancreas-specific mQTL. The top SNP-probe pairs in our study were not among the most significant hits reported in Olsson et al.¹⁵ However, pancreas mQTL was performed in healthy tissue and the cutoff for significance was conservative, based on the large number of CpG SNP combinations considered. It is possible that moderate effects would be detected in a more targeted testing framework. To evaluate the potential overlap between studies, we reviewed probes associated with rs689 in our mQTL as well as in the pancreas mQTL. We focused on the rs689 SNP based on its relevance to T1D and because probes associated with rs689 were also significantly related to T1D, making it a strong candidate for follow-up studies. In Olsson et al,¹⁵ the rs689 was associated with the cg16711450 and the cg22068589 probes. In our analysis, cg22068589 probe did not pass QC checks. The rs689 was associated with cg16711450 in our discovery analysis (nominal discovery P value = $2.25E-04$), but did not meet criteria for statistical significance. Furthermore, cg22068589 was located less than 1 KB from probes in our study that were related to rs689 and T1D (cg21574853 and cg25336198). Overall, the consistent pattern at these probes illuminates an area of the genome that may have an important role in T1D process and, thus, may be a suitable target for follow-up studies.

Previous publications from DAISY have identified significant associations between several non-HLA SNPs and T1D endpoints. However, the functional implications and/or mechanisms underlying these associations are unknown. Among methylation measurements obtained prior to T1D, we identified 10 significant CpG SNP pairs that were confirmed in the validation EPIC set. Many of the probe pairs map to regions with potential functional implications (Table S2) that should be targeted in follow-up studies aimed at understanding the consequences of allele specific methylation on the transcriptome, proteome, and ultimately the development of T1D. More importantly, three of these probes located near the *INS* gene were significantly related to progression to T1D and were consistent with a previous mQTL analysis¹³ that identified an association between rs689 and methylation levels near *INS*. The results suggest that methylation levels are present early in the disease process, even before the onset of IA. Further mechanistic studies are needed to understand how the SNP-methylation pairs identified in the current study influence biological processes such as gene expression and how these changes influence immune regulation and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

A-C, Box plots of genotype-methylation and methylation-phenotype association after the onset of IA. Description: Methylation levels obtained after the onset of islet autoimmunity and before the diagnosis of T1D in the cases by genotype and phenotype (T1D case vs T1D control). Methylation levels (% methylation) by rs689 genotype at the cg21574853 (A), cg03366382 (C), and cg25336198 (C) probes. Percent methylation at the cg21574853 (B), cg03366382 (D), and cg25336198 (F) probes in T1D cases vs T1D controls. Red and black dots represent methylation data from the EPIC and 450 K platforms, respectively

FIGURE 2.

Box plots of methylation-phenotype association before the onset of islet autoimmunity.

Description: Percent methylation at the cg21574853 probe in T1D cases vs T1D controls prior to onset of islet autoimmunity. A, cg21574853; B, cg03366382; and C, cg25336198 probes. Red and black dots represent methylation data from the EPIC and 450 K platforms, respectively

TABLE 1

Demographics in T1D cases vs controls

	Discovery (450 K)			Replication (EPIC)		
	T1D cases n = 39	Controls n = 39	P value	T1D cases n = 44	Controls n = 44	P value
First degree relative with T1D, n (%)	30 (77)	27 (71)	.4438	22 (50)	16 (36)	.1966
Non-Hispanic White, n (%)	37 (95)	36 (93)	>.999	36 (82)	36 (82)	>.999
DR3/4 genotype, n (%)	19 (49)	3 (12)	<.0001	22 (50)	11 (25)	.0154
Female, n (%)	16 (41)	14 (34)	.6416	25 (57)	20 (45)	.2863
Age at methylation visit, mean (SD)	5.1 ±3.9	5.0 ±3.6	.9852	3.9 ±3.4	3.5 ±3.3	.5915

TABLE 2
Results of the mQTL analysis including the discovery (450 K) and validation (EPIC) *P* values

Probe	SNP probe distance (KB)	Regulatory feature group	Relation to island	FDR adj. <i>P</i> value (450 K)	<i>P</i> value (EPIC)
rs2290400–GSDMB–Chromosome 17					
cg18711369 ^a	15	Promoter associated	N Shelf	1.47E-02	3.27E-04
cg24910161	53		Open Sea	3.57E-02	1.88E-06 ^b
rs229541–CIQTNF6–Chromosome 22					
cg02332492	102 249		S Shore	1.68E-02	4.34E-01
cg05209515	6		Open Sea	3.00E-03	1.69E-07 ^b
rs4788084–IL27–Chromosome 16					
cg06941159 ^a	335	Promoter associated	Island	3.96E-06	1.11E-08
cg09046979	207	Unclassified	S Shore	1.01E-06	2.01E-10 ^b
rs689–INS–Chromosome 11					
cg033366382	<1		Open Sea	2.61E-05	1.42E-15 ^b
cg08005462	30		Open Sea	1.33E-02	1.31E-06 ^b
cg08241307	30		Open Sea	1.42E-02	1.64E-07 ^b
cg19586845 ^a	30		Open Sea	1.44E-03	NA
cg21574853	<1		Open Sea	8.02E-08	1.23E-16 ^b
cg24338752	<1		Open Sea	8.42E-07	NA
cg25336198	<1		Open Sea	2.06E-04	1.65E-15 ^b

^aKnown SNP located in or near CpG probe, NA = not available for testing on the EPIC platform

^bSignificance (EPIC *p* value < .05) confirmed in cross-validation, considered candidate in DMP analysis (note: probes in known SNPs were not considered for the DMP analysis).

Association between methylation levels after the onset of islet autoimmunity and odds of progression to T1D

TABLE 3

Probe	450 K		EPIC		Meta-Analysis	
	OR	P value	OR	P value	OR	P value
cg03366382	2.02	.13294	1.77	0.19995	1.88	.0495
cg05209515	2.33	.31262	1.15	0.87312	1.65	.4045
cg08005462	0.79	.58834	0.63	0.29368	0.71	.2623
cg08241307	0.85	.49346	0.64	0.08564	0.74	.0958
cg09046979	1.64	.35411	0.79	0.68862	1.18	.6761
cg21574853	3.18	.08854	2.22	0.11189	2.53	.0232
cg24910161	1.43	.5809	1.26	0.76153	1.36	.5370
cg253336198	7.37	.06377	6.10	0.05841	6.62	.0081