Published in final edited form as:
JAMA. 2008 June 25; 299(24): 2877–2883. doi:10.1001/jama.299.24.2877.

Intra-individual change in DNA methylation over time with familial clustering

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Abstract

Context—Changes of epigenetic marks over time may help explain the late onset of common human disease.

Objectives—To determine whether there are longitudinal changes of global DNA methylation in individuals, and to evaluate whether methylation maintenance demonstrates familial clustering.

Design, Setting and Participants—Changes in HpaII methylation over time in a given individual have not yet been investigated. Here we measured global DNA methylation quantitatively by LUminometric Methylation Assay (LUMA), a quantitative measurement of genome-wide DNA methylation, on DNA sampled at two visits on average 11 years apart in an Icelandic cohort (1991 and 2002-2005) and on average 16 years apart in a Utah sample (1982-1985 and 1997-2005).

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Author contributions: Drs Bjornsson, Feinberg, Fallin, Irizarry, Aspelund, Gudnason, and Feinberg had full access to all of the data in the study, stripped of patient identifiers, and take responsibility for the integrity of the data and the accuracy of the data analysis. HTB and APF planned and organized the study and wrote the paper with MDF; MIS performed LUMA and computational analysis; MDF, RAI and TA developed and performed statistical analysis; HC modified LUMA with insights from TJE; WY performed cell fractionation; HTB and MR performed Illumina assays; CS and MFL developed and provided samples from the Utah cohort, and similarly TBH (an NIH sponsor), LJL (an NIH sponsor), GE, and VG from the Icelandic cohort; HTB, VG, and APF conceived the study. All authors approved the manuscript. H.T.B.., M.I.S., M.D.F., R.A.R., T.A., M.F.L., C.S., V.G., and A.P.F. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis

Main Outcome Measure—Global methylation changes over time.

Results—Twenty-nine percent of Icelandic individuals showed >10% methylation change over time ($p<0.0001$). The family-based Utah sample also showed intra-individual changes over time, and further demonstrated familial clustering of methylation change ($p=0.003$). The family showing greatest global methylation loss also demonstrated the greatest loss of gene-specific methylation by a separate methylation assay.

Conclusions—These data indicate that methylation does change over time and suggest that methylation maintenance may itself be under genetic control.

Unstructured Abstract—Epigenetic marks are modifications of DNA or associated proteins, other than the DNA sequence, that are heritable during cell division. Since the DNA remains the same, but epigenetic marks change over one's lifetime, changes of epigenetic marks over time may help explain the late onset of common human disease. Here we determined whether there are longitudinal changes of global DNA methylation in individuals, and evaluated whether methylation maintenance demonstrates familial clustering. We examined an Icelandic cohort sampled at two visits 11 years apart on average, and found that 29% changed >10% in their DNA methylation level. A second Utah cohort sampled at two visits 16 years apart on average showwed similar changes over time, with familial clustering. Thus, DNA be under genetic control.

Epigenetic marks are modifications of DNA or associated proteins, other than the DNA sequence itself, that are heritable through cell division. These include DNA methylation, a covalent modification of cytosine, histone modifications affecting the nucleosomes around which the DNA is coiled, and alterations in nucleosomal packing or higher order folding of chromatin. We and others have suggested that epigenetics might play a role in the etiology of common human diseases[1-3]. In a recent review in *JAMA* by one of us, we noted that epigenetics stands at the epicenter of modern medicine, because it unites nuclear reprogramming during development, environmentally induced changes on the body, and the ability of cells to respond appropriately to external stimuli[4]. That is because epigenetic changes, unlike the DNA sequence, distinguish one tissue type from another, and dietary and other environmental exposures alter the epigenetic program, the ability of genes to alter their expression is controlled by epigenetic factors such as DNA methylation[5, 6]. Diseases in which epigenetic change has been shown to play a major part include cancer and some disorders of the immune system, and epigenetic defects may also contribute to chronic diseases such as diabetes, bipolar disorder and autism, and loss of normal responsiveness to stress that accompanies aging[7-9].

A critical underpinning of the epigenetic hypothesis of common disease is that epigenetic marks change in the same individual over time. Recently, 14 monozygotic twins of 40 pairs tested were retrospectively found to be discordant in the degree of total DNA methylation and histone acetylation, with a preponderance over age 28 (ref. 10). In contrast, a recent analysis of ~1 Mb of genomic DNA encompassing 40,000 CpG dinucleotides found no difference in DNA methylation related to age, although those data were based on average values, rather than paired sampling in the same individuals over time[11]. To prove that epigenetic marks change in an individual a prospective study design is needed, and the only such study to date found no consistent methylation changes at two individual loci[12]. We have now performed a direct examination of methylation in the same individuals over time to resolve this important question.

METHODS

Samples

Icelandic samples were from the AGES Reykjavik Study, which is described in detail elsewhere[13]. In brief, the AGES study constitutes visit 7 (in 2002-2005) of the Reykjavik Heart Study, which began with 18,000 residents of Reykjavik recruited in 1967. The AGES study

recruited 5758 of the surviving members, who were aged 69–96 years in 2002. Of these, 638 gave a DNA sample in 1991 as part of the sixth Reykjavik Study visit, and therefore have DNA from two timepoints available for methylation analysis. The 111 analyzed here represent a sample of 61 individuals from the 638 with the largest amount of DNA in the study repository as well as an additional 50 chosen to represent surviving (all-cause) cancer cases within the cohort and with the largest amount of DNA in the repository. These samples are 50% male with an average age at first sampling of 74.6 [SD 2.9, range 70-82] years and time between sampling an average of 11 years. These 111 are not statistically significantly different from the 638 samples with two DNA visits on cholesterol, triglycerides, CRP levels, blood pressure, smoking, CHD, diabetes, or stroke prevalence, as of the last visit. They are statistically significantly older (by 0.6 years), however this rounds to the same age (84 in both groups at second visit). The intra-individual change in methylation over time was not statistically significantly different between samples from (all-cause) cancer and non-cancer samples.

Utah samples were from the Salt Lake City CEPH pedigrees collected between 1982 and 1984 (680 individuals from 48 three-generation families) as described[12]. As these were family sets, there was a broad range of ages, 5-72 years, with time between sampling an average of 16 years. All families were recontacted and 25 agreed to participate in a second sample collection, of whom 21 had sufficient DNA at both time points for more than one family member to be included in our analysis. Disease status was not a consideration in selection of individuals or families for analysis of longitudinal methylation changes. Neither sex ratio nor family size were significantly different (X^2=0.011; n.s.) between the collection of families analyzed in this report (mean of 7.1 females and 7.5 males per family) and the families on whom collection of second blood DNA samples has not yet been obtained (mean of 7.2 females and 7.5 males per family).

All DNA was from unfractionated peripheral blood cells (non-transformed cells). Institutional informed consent was obtained in writing from all participants. This methylation study was reviewed approved by the appropriate IRBs at the University of Utah, the Icelandic National Bioethics Committee, and the Johns Hopkins Bloomberg School of Public Health. All UGRP study participants gave informed consent under University of Utah IRB approved protocol number 6090-96. Participant recruitment in the AGES cohort (Iceland) and sample sharing for this project were approved by the Icelandic National Bioethics Committee (FS-04-001). For cell fractionation, the Dynabeads (Invitrogen, Carlsbad, CA) were used on buffy coats isolated using Ficoll-Paque Plus (GE-Healthcare, Piscataway, NJ).

Modification of the LUMA protocol

The LUMA protocol has been described in detail previously[14]. We modified the protocol to minimize effects of degradation on quantification by adding additional measurements for free DNA ends. Details are available on request. We performed a mixing study using predetermined proportions of either fully methylated (SssI methylase) or unmethylated Lambda phage DNA and measured the proportions utilizing LUMA, using this standard curve to convert HpaII/MspI ratios into HpaII methylation. The assay was linear at the range of 0-100% methylation (R^2=0.984, R=0.992). Furthermore, the assay was validated by demonstrating the marked hypomethylation found in the previously described DNA methyltransferase I double knockout (DKO) cell line compared to the parent cell line (HCT116). Experiments with three separate digestions and measurements on 25 samples revealed that the average variance of the assay was 2%. Measurements of HpaII methylation of whole blood from 7 individuals sampled 2-4 times over a period of 30 days confirmed the stability of methylation in both total buffy coat WBC DNA as well as fractionated T cells, the predominant cell population,,showing no significant change in methylation. Furthermore, repeat assays performed 1 year later on the

original samples from the 9 individuals showing the greatest difference in methylation between timepoints from the Icelandic cohort confirmed our initial measurements.

DNA methylation analysis

DNA (0.5 μg) was bisulfite treated with the EZ DNA methylation kit (Zymo Research, Orange, CA). DNA methylation analysis of individual genes was performed using the commercially available Illumina GoldenGate Methylation Solution[15], utilizing the current Cancer Panel I platform (Illumina, San Diego, CA), which probes 1,505 CpG loci selected from 807 genes. The Illumina assay has been validated by both bisulfite sequencing and methyl specific PCR[15] as well as by quantitative bisulfite pyrosequencing[16].

Statistical analysis of LUMA data

To assess the statistical significance of intra-individual changes in methylation over time in the Iceland sample, we performed permutations of the time labels to generated random draws from the null hypothesis of no time effects. In our case, there were three measurements at time 1 and three measurements at time 2 for each individual. When there is no true change over time, these 6 values should be estimates of the same underlying value and simply reflect random and measurement error. Therefore, at each permutation, we shuffled these 6 measurements within individuals and randomly assigned three to time 1 and the other three to time 2. We then averaged the 3 values for each permuted time point and then calculated the difference between time 2 and time 1. To estimate how likely our observation is to be due to chance if there are no true changes in methylation over time, we also calculated the ratio of variance in methylation across all 6 measures over the variance within each time point ($R=Var^{between}/Var^{within}$). We compared the ratio in our observed data (R=11.23) to the distribution of R values from each of 10,000 permutations. None of the 10,000 showed an R as extreme as that observed (P value < 0.0001). These analyses were performed in SAS v9.1.

To estimate heritability of methylation change in the Utah family sample, we first calculated the change between time 2 and time 1, adjusted for time 1 values, to accommodate the influence of time 1 values on the amount of change possible. Residual values for each sample after regressing methylation values at time 2 onto those at time 1 were then used to calculate maximum likelihood estimates of heritability, using variance components models in the ASSOC program of the S.A.G.E. package[17].

Statistical analysis of Illumina data

To assure readings from different samples were comparable for the Illumina gene-specific dataset, we quantile normalized the data in the following way: we combined the red/cy5 (methylated) and green/cy3 (unmethylated) intensities into one vector that should be proportional to the amount of DNA in the sample. Because these amounts should be the same we quantile normalized these vectors[18], then separated them back into individual intensities. We then formed log ratios which should be proportional to the log proportion of methylated targets, and which made these values symmetric and close to normally distributed across samples for a given probe. To assess the consistency in changes in methylation across time-points for family 21, we computed a t-test for the difference in log-ratios for individuals. To assess the size of the change, we back-transformed the log ratios to percentages (red / (red + green)) as done by Illumina[15] and computed the differences in percentages in methylation. To quantify changes we found it was important to look at differences in percentages instead of differences in log ratios because a change from, for example 0.1% methylation to 1% methylation, while 10-fold, is not likely to be biologically meaningful.

RESULTS

General strategy

We have examined the question of time-dependent epigenetic changes directly by the luminometric methylation assay (LUMA), a global measure of Hpa II/ Msp I methylation, which cleaves 5-'CCGG-3′ sites that are, respectively, dependent and independent of methylation of the internal cytosine. The higher this ratio, the more methylated the DNA template is. We first performed an *in silico* analysis of the method, mapping all Hpa II sites in the human genome, which showed 15-fold enrichment of CpG islands, potentially important regulatory sequences.. We also modified LUMA to minimize the possibility of error due to DNA degradation (see Methods).

Changes in global methylation marks in an Icelandic cohort

We first analyzed DNA from 111 participants in the AGES Reykjavik Study[13]. The sorted change values are shown in red in **FIGURE 1.** While the mean intra-individual difference between Hpa II methylation over an average of 11 years was 0, a wide range of changes were observed (min= -0.30, max = 0.26), with 70 (63%) individuals showing a change of at least 0.05 in either direction, 33 (30%) individuals showing a change ≥0.10, and 9 (8.1%) showing changes ≥0.20. Since roughly the same number of participants showed a decrease in methylation as an increase,, this substantial intra-individual change would likely be missed by age-specific cross-sectional analysis. To gauge whether the differences observed were due to measurement variation or chance, we performed permutations of the data to simulate no change over time (**FIGURE 1.**) None of the 10,000 permuted data sets showed a distribution of change in methylation over time as extreme as the observed data ($P < 0.0001$). In addition, the coefficient of variation (CV) for within subject triplicate measures of LUMA was only 2.4% compared to the CV across individuals of 10.2%. Inflammatory markers, such as ESR, CRP and white blood count, were also available for these participants at both visits (measured by Westergren method, Hitachi 912, and Coulter Counter, respectively). These measures showed no relationship to DNA methylation levels, defined as the residual after adjustment of the change in methylation by the time 1 value, to accommodate any dependency on time point 1 values, indicating that the methylation changes were not due to an inflammatory state or redistribution of white blood cells. Further, age and length of storage were not correlated with change in methylation. The length of time between measures was slightly correlated with change (in either direction, using the absolute value of the residual)(ρ=.14), although this was not statistically significant ($p = .17$). This trend may be expected if methylation is indeed changing over time in individuals.

Changes in global methylation marks in a second familial based cohort

To confirm these results, we examined DNA from a second cohort of 126 individuals from a collection of Utah pedigrees that had been sampled twice over an average of 16 years. Like the Icelandic population, a wide range of changes were observed in this sample as well (min=-0.49, max = 0.39), with 50 (40%) individuals showing a change of at least 0.05, 23 (18%) showing a change ≥ 0.10 and 13 showing changes ≥ 0.20 between time points.

An additional advantage of the Utah cohort is the inclusion of families, allowing estimation of familial correlations in methylation change over time. Many showed clustering of methylation change in most or all family members (**FIGURE 2**). Interestingly, this clustering occurred for both decreased and increased methylation. In general, the familial correlations in methylation were more striking at time point 2 compared to time point 1, indicating that the differences were not due to acquired instability of the DNA due to longer storage, which we also demonstrated directly (see Methods). While shared family environment could explain this clustering, most families contained two generations of adults (i.e., the average ages of offspring

sampled at time points 1 and 2 were 17 and 32 years, respectively), who likely did not share households during the majority of the time in between samplings. This suggests that the stringency of global methylation pattern maintenance is itself a heritable trait. Such familiality was most striking for decreased methylation, since 7 of the 13 most extreme decreases in methylation over time were within 2 families, families 21 and 9 (**FIGURE 2**). To assess the significance of this clustering, we calculated the heritability of methylation change based on these family data. To account for any correlation between time 2 and time 1 LUMA values, we adjusted the change in methylation by the time 1 value, and used each subject's residual value as the phenotype for heritability analysis. The heritability estimate was .99, $p < .0001$. This familiality was not limited to a single family and remained statistically significant ($h^2 = 0.743$, $p = .0026$) after removal of family 21. This suggests that, although family 21 is clearly an outlier in the amount of change, and the tight clustering among family members, the rest of the families still show clustering within versus across families, support a heritable component to methylation stability.

Gene-specific methylation studies confirm the global methylation findings

In order to gain some insight into gene-specific methylation changes, we examined a panel of 1,505 CpG dinucleotides in 807 genes (~3% of known human genes) using the recently released GoldenGate methylation assay[15] (cancer panel 1). We analyzed a subset of 41 individuals at each of two time points, representing 17, 5, and 19 individuals showing the greatest loss, least change, or greatest gain, respectively, in global DNA methylation as measured by LUMA. When individuals were analyzed based on the difference in methylation over time (distance computed based on all genes tested), there was tight clustering of family 21 ($P < 10^{-8}$), which also showed the greatest change by LUMA. Furthermore, of the 50 CpG probes that showed the greatest change over time in the 5 members of family 21, 49 showed methylation loss with age ($P < 10^{-16}$, by chance expecting 25; **TABLE 1**). There was a small but statistically significant enrichment for CpG probes within imprinted genes in this subset of genes (5 of 50, compared to 28 of 807 on the array, $P < 0.05$). Furthermore, comparing the 50 CpGs with the greatest difference across all individuals (not in family 21), 13 were shared with family 21, which was highly statistically significant ($P < 10^{-16}$; **TABLE 2**). There were a number of immunological mediators among these genes (**TABLE 2**), which is intriguing given the suggested role of altered DNA methylation in immunological disease[19, 20]. Furthermore, among the genes that changed the most, family 21 had more genes in common with the most extreme outliers from families 3 and 9 than would be expected by chance alone.

COMMENT

In summary, we have observed time-dependent changes in global DNA methylation within the same individual, in two separate populations in widely separated geographic locations, with 8-10% of individuals in both populations showing changes >20% over an 11-16 year time span. These changes showed familial clustering of both increased and decreased methylation, and were most marked (>30%) in a family with 5 individuals showing loss of methylation over time, and in whom methylation alterations were confirmed by examination of ~1500 CpG dinucleotides in 807 arbitrarily selected genes. The enrichment for imprinted genes was intriguing given the sensitivity of imprinted genes to both *in vitro* fertilization in humans[21] and dietary modification in mice[22]. These data support the idea of age-related loss of normal epigenetic patterns as a mechanism for the late onset of the common human diseases (common disease genetic and epigenetic model)[1], which could arise through the loss of functionally important epigenetic modifications as well as through the release of epigenetic buffering of intrinsic genetic variation[23, 24]. In that regard, it is particularly interesting that many of the genes showing common variation are involved in immune system modulation, and thus might reflect temporally acquired changes in the cell type that was studied (lymphocytes). However,

lymphoid tissues might also act as a good surrogate tissue for changes in other target tissues as for loss of imprinting (LOI) of IGF2, one of the best studied epimutations, the defect is found in both lymphocytes as well as colon and changes of either are associated with increased colorectal cancer risk[25]. The familial clustering of methylation changes also raises the possibility that methylation changes also raises the possibility that methylation stability might be directly related to genetic variation, such as in genes controlling one-carbon metabolism or DNA methyltransferase activity. Consistent with this idea, gene-environment interactions affecting interactions affecting folate biosynthesis are linked to risk of colorectal neoplasia[26]. The mechanism could involve altered methylation of specific genes, such as that leading to loss of imprinting of *IGF2* associated with colorectal cancer risk[27]

We observed both losses and gain of DNA methylation over time in different individuals, and both could contribute to disease, which subsequent studies will need to determine. For example, cancer is associated with both hypomethylation and hypermethylation, through activation of oncogenes and silencing of tumor suppressor genes, respectively [28]. Similarly, animal studies have shown that a loss of DNA methylation increases intestinal adenoma initiation and a gain of DNA methylation increases adenoma progression[29]. Similarly, both hypomethylation and hypermethylation could lead to autoimmune disease by activating autoreactivity genes or silencing histocompatibility genes[19, 30]. Our data stand in contrast to the observation of Eckhardt *et al.*[11] that there are no changes in DNA methylation over time. In that study, values were averaged across individuals for a given age group, while our data suggest considerable inter-individual age variation with differences accruing over time within individuals that would be missed by group averaging.

Finally, we note that the implications of these results are potentially profound for population-based studies of human disease. It shows that the epigenome changes in individuals over time, which might directly influence disease phenotype. Epigenetic changes may also might reflect age-related or environmental exposures. Thus, including epigenetic measurements in epidemiological studies could open a molecular window into potential genome exposures as well as mechanisms.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant P50-HG003233 (A.F.), NIH Grant R01-ES015211 (M.D.F), the Swedish Cancer Foundation (T.J.E.), NIH contract N01-AG-12100 (V.G., T.H., L.L.), the Icelandic Parliament (V.G.), Huntsman GCRC grant M01-RR00064 (M.L.), and the W.M. Keck Foundation and George S. and Delores Doré Eccles Foundations (M.L.). H.B. was supported by the Fulbright Foundation, and M.I.S. was supported by a grant from the Icelandic Student Innovation Fund (2006). We thank Andreas Peiffer, M.D. and Melissa M. Dixon, M.S., University of Utah, for technical assistance.

TBH and LJL are NIH sponsors and assisted in developing the Icelandic cohort. There are no other conflicts of interest or relevant financial relationships or affiliations for any of the authors.

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Table 1

The 50 genes that showed the greatest change over time in the 5 members of family 21. Of the 50 genes, all but one (*LIF*) demonstrated a decrease in methylation between the two time points.

Gene name	Chromosome	Difference[a]	P value[b]
PWCR1[c]	15	-0.723	0.000
IL1B	2	-0.420	0.001
KCNK4	11	-0.402	0.001
AIM2	1	-0.372	0.000
PI3	20	-0.306	0.003
CSF3R	1	-0.301	0.010
GLA	X	-0.274	0.001
PLA2G2A	1	-0.259	0.007
NOTCH4	6	-0.255	0.001
TRPM5[c]	11	-0.251	0.001
HDAC6	X	-0.247	0.003
GFAP	17	-0.246	0.000
HOXA5	7	-0.242	0.011
PTK6	20	-0.226	0.014
G6PD	X	-0.210	0.017
ELK1	X	-0.205	0.005
G6PD	X	-0.204	0.041
ERCC3	2	-0.202	0.063
LMO2	11	-0.201	0.021
CSF2	5	-0.200	0.001
LIF	22	0.200	0.006
ELK1	X	-0.195	0.003
PLG	6	-0.190	0.001
ARAF	X	-0.180	0.004
DKC1	X	-0.180	0.190
G6PD	X	-0.177	0.005
FMR1	X	-0.177	0.023
BCAP31	X	-0.174	0.002
SNRPN[c]	15	-0.172	0.001
BAX	19	-0.170	0.001
SYK	9	-0.169	0.013
GLA	X	-0.169	0.007
VBP1	X	-0.168	0.006
IL10	1	-0.168	0.055
LMO2	11	-0.163	0.076
MPL	1	-0.162	0.012
TRIP6	7	-0.162	0.037
IRAK1	X	-0.160	0.068
VBP1	X	-0.158	0.001
BIRC4	X	-0.155	0.027
SLC22A18[c]	11	-0.154	0.016
LCN2	9	-0.152	0.007
SLC22A2[c]	6	-0.152	0.002
IL16	15	-0.151	0.012
SNCG	10	-0.142	0.110
LCN2	9	-0.142	0.110
DNASE1L1	X	-0.142	0.014
EMR3	19	-0.138	0.004
ELK1	X	-0.138	0.059
DNASE1L1	X	-0.135	0.076

[a] Fractional difference in DNA methylation (time point 2, minus time point 1, with 0-1 representing none to completely methylated at each time point; a negative value indicates a loss of DNA methylation in an individual over time)

[b] T test.

[c] Imprinted genes

Table 2

Genes showing greatest differences in family 21 and also across all individuals, showing a high representation of immunological mediators among these genes.

Gene name	Gene function
AIM2[a]	Interferon gamma inducible transcript
CSF3R[a]	Colony stimulating factor 3 receptor
HOXA5	Hox gene
PTK6	Protein tyrosine kinase
ERCC3	Helicase with excision-repair functions
LMO2[a]	Role in erythropoiesis and in T-cell leukemogenesis
SYK[a]	Spleen-tyrosine kinase
IL10[a]	Cytokine
BIRC4	Apoptosis inhibitor
IL16[a]	Cytokine
LCN2[a]	Protein associated with neutrophil gelatinase
TRIP6	Regulates lysophosphatidic acid induced cell migration
EMR3[a]	Myeloid-myeloid interactions during immune and inflammatory responses

[a]Genes that play a role as immunological modulators