Research Paper

DNA methylation clocks as a predictor for ageing and age estimation in naked mole-rats, *Heterocephalus glaber*

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ABSTRACT

The naked mole-rat, *Heterocephalus glaber* (NMR), the longest-lived rodent, is of significance and interest in the study of biomarkers for ageing. Recent breakthroughs in this field have revealed 'epigenetic clocks' that are based on the temporal accumulation of DNA methylation at specific genomic sites. Here, we validate the hypothesis of an epigenetic clock in NMRs based on changes in methylation of targeted CpG sites. We initially analysed 51 CpGs in NMR livers spanning an age range of 39-1,144 weeks and found 23 to be significantly associated with age ($p<0.05$). We then built a predictor of age using these sites. To test the accuracy of this model, we analysed an additional set of liver samples, and were successfully able to predict their age with a root mean squared error of 166 weeks. We also profiled skin samples with the same age range, finding a striking correlation between their predicted age versus their actual age ($R=0.93$), but which was lower when compared to the liver, suggesting that skin ages slower than the liver in NMRs. Our model will enable the prediction of age in wild-caught and captive NMRs of unknown age, and will be invaluable for further mechanistic studies of mammalian ageing.

INTRODUCTION

The naked mole-rat (*Heterocephalus glaber*) is arguably unique among mammals in the extent of its social insect-like behaviour, first highlighted by Jarvis [1]. In more recent years it has also gained prominence as an important non-model organism for the study of longevity [2–5], and other extraordinary aspects of its biology that result from adaptations to the challenges of an extreme subterranean niche [6, 7]. Although little more than mouse-sized (mean body mass is around 35g), naked mole-rats are the longest-lived rodent with a maximum possible lifespan exceeding 31 years [5]. Furthermore, they resist all of the normal signs of ageing and are thus emerging as an important non-model organism for the study of longevity and healthspan. These unusual mammals uniquely do not show an increase in age-specific hazard of mortality, in defiance of Gompertz's law [8]. They also show no decreased physiological capacity with age, maintaining vascular elasticity, cardiac function, gastrointestinal function, glucose tolerance, and reproductive capacity

well into the third decade of life [2, 9, 10], and resist sarcopenia, the progressive loss of skeletal muscle with age [11]. Because of these traits, the naked mole-rat is of particular significance and interest in the study of biomarkers for ageing. Recently, breakthroughs in this field have indicated the presence of 'epigenetic clocks', mainly in studies of human and mouse tissues [12–15], but also in canids [16, 17], and humpback whales [18]. These are based on the temporal accumulation of DNA methylations at specific ageing-associated differentially methylated positions (aDMPs). Such CpG sites at which DNA methylation dynamics show significant correlations with age can potentially enable accurate age estimates for tissues across the life span of an individual, and it has been shown in humans that most tissues and organs from the same body exhibit broadly similar epigenetic ages [19].

In an analysis of aDMPs in six different mammals, including long and short-lived dog breeds, Lowe et al. [17] found a strong negative relationship between rate of change of methylation levels at aDMPs and lifespan. This study also identified 30 aDMPs in the naked mole-rat liver, that clustered in 12 different targeted aDMP regions, providing a potential molecular readout for aging in this species. A challenge for research on long-lived non-model organisms such as the naked mole-rat, especially when studying wild caught animals, is the determination of age (in the absence of birth and life history data). In some cases, this problem also applies to captive populations where pedigree data is not available. Crude categorical estimates are sometimes possible based on tooth wear, for example in other African mole-rats (*Cryptomys* and *Fukomys*; [20]), but these only differentiate relative age classes, rather than attempting to assign an absolute age estimate. Here, we aim to (i) consolidate and validate these initial results for the naked mole-rat, examining further samples across a wide age range in both liver and skin, and (ii) develop a method that will enable naked mole-rat aDMPs to be used to estimate age in animals of unknown provenance.

RESULTS

To create a method for predicting the age of naked mole-rats based on changes in methylation, we initially sampled 24 naked mole-rat livers spanning an age range from 39 weeks to 1,144 weeks (approximately ten months to 22 years; Supplementary Table 1). We performed a targeted sequence-based method to determine the methylation of individual CpGs across the genome. These targeted regions were selected by mapping existing regions within the human genome, known to be associated with age, to the nakedmole-rat genome (https://www.ncbi.nlm.nih.gov/pubmed/25172923). In total we selected 12 regions/primer pairs spanning a total of 51 different CpGs (Supplementary Table 2). Of these 51 different sites 23 (45%) were found to be associated with age (p-value < 0.05; Supplementary Table 3). The top hit (JH602136:8746439) showed a strong correlation (R=0.88) with age (Figure 1A) with a root mean square error of 541.95 weeks.

Utilising a similar methodology to Horvath [19] in producing his multi-tissue age prediction in humans, we built a predictor of age using the 23 sites that showed an association with age in the naked mole-rat. This uses elastic net regression which incorporates all 23 CpGs in a multivariate analysis. To validate this model, we performed a leave-one-out cross validation in which we remove a single sample to fit the parameters of our model and then predict the age of this single sample. We then repeated this until we had removed each sample and predicted its age. Utilising multiple CpGs in the model showed a slight improvement in correlation (0.89) but a large decrease in the RMSE (134.21) (Figure 1B). To further test whether this model could be useful, we sampled a further 19 livers with an age range 43 to 1,196 weeks (approximately ten months to 23 years; Supplementary Table 1). Using the model built from the initial 24 samples, we predicted the age of these new samples. As expected, given this was a different batch of samples both the R and RMSE dropped albeit only by a small amount (Figure 1C). Given the multi-tissue nature of some methylation changes within humans, we decided to test our model using a different set of tissue samples. We profiled a further 20 skin samples with the same age range of that of the 19 liver samples (19 of the 20 skin samples were from the same animals as the livers). Interestingly we found a striking correlation between the predicted age of these samples versus their actual age (R=0.93), however, this correlation was not along the identity line but showed a much lower predicted age than actual age suggesting that skin tissue ages slower than the liver. This change in rate accounts for the larger RMSE. To make this approach useable for other researchers we have provided the primer sequences that span the CpGs, and an online tool that can be downloaded as an NMR age predictor based on these aDMPs (https://github.com/ralowe/NMRAgePrediction; Supplementary Material).

DISCUSSION

In an extensive study of naked mole-rats, we identify 23 CpGs in liver tissue that are significantly associated with age, and build a predictor of age model with a root mean squared error of 166 weeks, or approximately 10% of the published maximum possible lifespan (more than 31 years/1612 weeks; [5]). In profiling skin samples from the same individuals, we also found a striking correlation between the predicted age of these samples versus their actual age. Interestingly, when

compared to the liver samples, this correlation showed a lower predicted age than actual age, suggesting that skin tissue ages more slowly than the liver in naked mole-rats. In humans, Horvath [19], found that most tissues from the same body exhibit broadly similar epigenetic ages. Other studies have showed that, while some age-associated changes in methylation are shared across tissues, others may be tissue-specific in humans [21, 22], mice (lung, liver, spleen and colon; [23]) and rats (liver and visceral adipose tissues; [24]). The relative proportions of age-associated epigenetic changes in the form of DNA methylation that are tissue-specific, compared to the amount that is general and non-specific remains a matter of debate. Zhu et al. [25] estimate that more than 70% is due to shared epigenetic drift across tissues, with the remainder down to tissue-specific and functionally important changes. The differences in ageing between liver and skin seen in the naked mole-rat may perhaps reflect the increased metabolic activity of the liver when compared to skin, and the fact that naked mole-rats are not exposed to UV radiation in captive or wild colonies. A variety of naked mole-rat tissues have been shown to resist the normal signs of senescence (see Lewis and Buffenstein [5] for review), including the liver where proteasome function increased or was maintained with age [26], and there was no accumulation of oxidative damage with age [27]. Although the ageing skin has not been investigated fully in naked mole-rats, their fibroblasts in culture are resistant to an array of toxins and stressors (compared

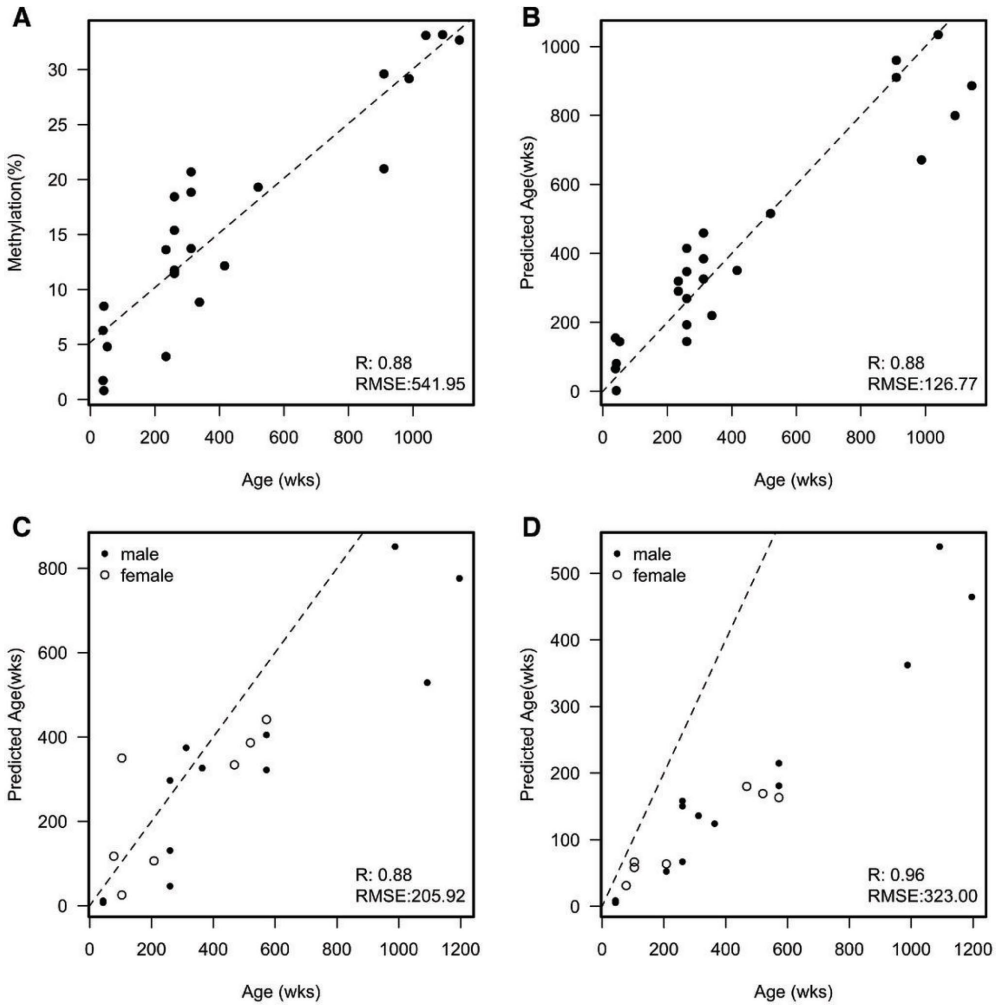

Figure 1. (**A**) Example of a single CpG that correlates with the age of each naked mole-rat. Dashed line is a fitted linear model; RMSE = Root Mean Square Error. Mean absolute deviation (MAD) = 412.282 and median absolute error (MAE) = 400.198; (**B**) A scatterplot of the predicted age of each naked mole-rat liver sample against the actual age in weeks from an initial sample set. The predicted age was calculated by removing the sample and fitting to the remaining samples. Dashed line represents y=x (e.g. perfect prediction). MAD = 96.882 and MAE = 120.840; (**C**) A scatter plot of the predicted age of a second set of naked mole-rat livers against the actual age in weeks. Dashed line represents y=x (e.g. perfect prediction). MAD = 118.941 and MAE = 126.883; (**D**) A scatter plot of the predicted age of skin samples against the actual age in weeks. Dashed line represents y=x (e.g. perfect prediction). MAD = 219.113 and MAE = 252.874.

with mice), including heavy metals, heat, and chemotherapeutic/DNA-damaging compounds [28, 29, 5]. Further, MacRae et al. [30] report that the naked mole-rat liver has higher expression of DNA repair genes, with significant upregulation of several DNA repair signaling pathways compared with the mouse. These observations of increased DNA repair in the naked mole-rat perhaps support the proposal of Field et al. [31], that long term maintenance of a steady state in dynamic chromatin ("chromostasis") may slow the ticking of the epigenetic clock in long-lived species.

Horvath [19] suggests that understanding how and why the estimated epigenetic age differs across a group of individuals of the same chronological age could help to determine the impact of endogenous or exogenous stress factors on biological ageing. Humans suffering from Werner's Syndrome, a condition that produces clinical signs of accelerated ageing also had associated epigenetic age acceleration and thus an increased DNA methylation age [32]. Other studies have shown that in humans, lifestyle factors including diet and physical activity, can have a positive association with epigenetic age acceleration i.e. a healthy lifestyle associates with a reduced epigenetic age [33]. Naked mole-rats are apparently unique among mammals in that they defy Gompertz's Law in not showing increased risk of mortality with age [8]. Furthermore, there are no apparent sex or reproductive status differences in their maximum possible lifespan [3]. It is interesting that some variance in the percentage methylation is evident for liver tissue in Figure 1A, perhaps suggesting some differences in biological versus chronological age among these mole-rat samples.

The potential for a forensic use for epigenetic clocks to determine the unknown age of a sample or individual has also been noted for humans by Wagner [34]. Our model and online tool will enable the prediction of age in wild caught naked mole-rats and captive animals of unknown age. Given the evidence for associations between epigenetic age acceleration and factors that may influence longevity in humans, and that most clocks have focused on application in humans [14] our study will be invaluable for further mechanistic and functional studies of mammalian ageing in non-model organisms such as the naked mole-rat.

MATERIALS AND METHODS

Naked mole-rats were maintained in the Biological Services Unit at Queen Mary University of London, and tissues obtained from post-mortem specimens from animals free from disease in compliance with national (Home Office) and institutional procedures and guidelines. Because sample collection was from post-mortem material, additional local ethical approval was not required for this study. Samples of abdominal skin and liver were snap frozen in liquid nitrogen and transferred for storage at -80ºC. Full details of animals and samples are provided in Supplementary Table 1.

DNA extraction

DNA was extracted from tissues using the PureLink™ Genomic DNA kit (Thermo Fisher, Cat. K182002), as per the manufacturer's instructions. Tissues were digested overnight at 55 °C using 180 µl PK buffer and 20 µl PK enzyme from the kit. DNA concentration was quantified using a High Sensitivity DNA Qubit® assay (Life Technologies, Cat. Q32851).

Bisulfite PCR sequencing (Bis-PCR-Seq)

DNA from tissues was diluted to a concentration of 11 ng/µl and 45 µl of each sample was used for generation of targeted bisulfite sequencing data by the Genome Centre Facility at the Blizard Institute, Queen Mary University of London. DNA was bisulfite converted in a 96 well plate format using the EZ-96 DNA Methylation™ Kit (Zymo, Cat. D5003). Target amplification was performed using the FastStart High Fidelity PCR System, dNTPack (Sigma-Aldrich, Cat. 4738284001) in the 48.48 layout on the Fluidigm® C1 system (Fluidigm®, USA), a microfluidics platform. Library preparation was performed using the same kit including 4 µl of Access Array Barcode Library Primer and 1 µl of PCR product diluted 1:100. Libraries were sequenced with Illumina MiSeq sequencing using v2 chemistry (150 bp, paired-end). Primers used for targeted bisulfite sequencing are listed in Supplementary Table 2.

Raw FASTQ files were mapped to the reference hetGla2 using BISMARK (v0.16.3) [35] and Bowtie2 (v2.2.8) [36]. Reads that mapped outside of the targeted regions were discarded from analyses, and methylated and unmethylated counts for each CpG were calculated using the custom C++ program (https://bitbucket.org/lowelabqmul/methylation-extractor/src/master/). Those CpGs with a coverage < 50× were also discarded from analyses. Data created for this manuscript are available from GEO with accession number GSE86059 (sample set 1) and GSE137957 (sample set 2), and provided in Supplementary File 1.

Statistical analysis

The analysis model used elastic net regression incorporating all 23 CpGs in a multivariate analysis. Linear models and scatterplots were produced using R statistical software [37]. We have developed an online

tool for analysis of similar NMR data (available at https://github.com/ralowe/NMRAgePrediction), with further information provided in the Supplementary Material).

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CONFLICTS OF INTEREST

There are no conflicts of interest to be declared.

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SUPPLEMENTARY MATERIALS

NMRAgePrediction tool

A downloadable software package enabling users to predict age from NMR data obtained using the methodology reported in this paper is available at: (https://github.com/ralowe/NMRAgePrediction

Full instructions on use is included in the README file. The tool requires Python version 3.7, pip, numpy, and scipy to be installed on the local machine prior to running NMRAgePrediction. The tool uses a model based on our animals/samples of known age to predict age, based on the proportion of methylated sites at the NMR aDMPs we describe. The input data should be expressed as methylation beta values, i.e. the ratio of methylated and unmethylated alleles expressed between 0 and 1.0.

Supplementary Tables

Supplementary Table 1. Sample details for the two sets of analyses, sample id corresponds to column headings in Supplementary File 1.

Sample (Set/id)	Anim/ No/	Colony	Age (yr)	Age (weeks)	Tissue
Set 1/a1	BM857	800	22	1144	Liver
Set 1/a2	M160	NN	20	1040	Liver
Set 1/a3	M224	Omega	17/5	910	Liver
Set 1/a4	M269	800	6	312	Liver
Set 1/a5	M576	800	5	260	Liver
Set 1/a6	M1	11A	0/8	42	Liver
Set 1/a7	M2	11A	0/8	42	Liver
Set 1/a8	M200	NN	19	988	Liver
Set 1/a9	M227	Omega	17/5	910	Liver
Set 1/a10	M065	800	5	260	Liver
Set 1/a11	M3	11A	1	52	Liver
Set 1/a12	M46	F(K)	0/75	39	Liver
Set 1/b1	M47	F(K)	0/75	39	Liver
Set 1/b2	M571	800	4/5	234	Liver
Set 1/b3	M795	800	4/5	234	Liver
Set 1/b4	M815	800	6	312	Liver
Set 1/b5	M025	B	5	260	Liver
Set 1/b6	M310	B	5	260	Liver
Set 1/b7	BM276	B	8	416	Liver
Set 1/b8	BM863	B	21	1092	Liver
Set 1/b9	BM555	11b	10	520	Liver
Set 1/b10	M056	Omega	6	312	Liver
Set 1/b11	M098	Omega	5	260	Liver
Set 1/b12	M033	B	6/5	338	Liver
Set 2/A3	F033	B	5	260	Liver
Set 2/A4	F033	B	5	260	Skin
Set 2/A5	F368	B	5	260	Liver
Set 2/A6	F368	B	5	260	Skin
Set 2/A7	F 279	800	5	260	Liver
Set 2/A8	F 279	800	5	260	Skin
Set 2/A9	F619	Omega	6	312	Liver
Set 2/A10	F619	Omega	6	312	Skin
Set 2/A11	F099	CF27	4	208	Liver
Set 2/A12	F099	CF27	4	208	Skin
Set 2/B1	BF336	11b	7	364	Liver
Set 2/B2	BF336	11b	7	364	Skin
Set 2/B3	BF7095	B	21	1092	Liver

Set 2/B4	BF7095	B	21	1092	Skin
Set 2/B5	F198	NN	23	1196	Liver
Set 2/B6	F198	NN	23	1196	Skin
Set 2/B9	M2	11C	1/5	78	Liver
Set 2/B10	M2	11C	1/5	78	Skin
Set 2/1B11	M191	N	4	208	Liver
Set 2/B12	M191	N	4	208	Skin
Set 2/C1	M775	G	9	468	Liver
Set 2/C2	M775	G	9	468	Skin
Set 2/C5	F353	11a	0/83	43	Liver
Set 2/C6	F353	11a	0/83	43	Skin
Set 2/C7	F046	11a	0/83	43	Liver
Set 2/C8	F046	11a	0/83	43	Skin
Set 2/C9	F581	G	11	572	Liver
Set 2/C10	F581	G	11	572	Skin
Set 2/C11	F264	G	11	572	Liver
Set 2/C12	F264	G	11	572	Skin
Set 2/D1	M285	11A	2	104	Liver
Set 2/D2	M285	11A	2	104	Skin
Set 2/D5	M612	11A	2	104	Liver
Set 2/D6	M612	11A	2	104	Skin
Set 2/D7	M353	CF05A	11	572	Liver
Set 2/D8	M353	CF05A	11	572	Skin
Set 2/D9	M124	CF05A	10	520	Liver
Set 2/D10	M124	CF05A	10	520	Skin
Set 2/D11	F10	Zoo	19	988	Liver
Set 2/D12	F10	Zoo	19	988	Skin

Animal number prefixes as follows: BM, breeding male; M, non-breeding male; F, non-breeding female; BF, breeding queen.

Supplementary Table 2. Primer sequences used for targeted assay of methylation.

Primer ID	Chr	Spos	Epos	Forward primer	Reverse primer
NMR 2	JH602048	1786748	1787040	ACACTGACGACATGGTTCTACAt gtgtgaaaaaTaagagtggtggt	TACGGTAGCAGAGACTTGGT CTAtttcctcccttcctcctAaca
NMR 4	JH602120	4015571	4015913	ACACTGACGACATGGTTCTACA gggaggaaggTttTagagatggg	TACGGTAGCAGAGACTTGGT CTAcaccttcctcaccccaAAca
NMR 5	JH602160	1882997	1883289	ACACTGACGACATGGTTCTACA ggtgggtggggtgaaagtag	TACGGTAGCAGAGACTTGGT CTAactccccaaccactaActcct
NMR 6	JH602123	5429096	5429239	ACACTGACGACATGGTTCTACA agagggtTaTatggagtggttTTT	TACGGTAGCAGAGACTTGGT CTAaccttTaattcaccaAaAccttttct
NMR 7	JH602136	8746392	8746577	ACACTGACGACATGGTTCTACA gagtTtgggtgggagTtgtT	TACGGTAGCAGAGACTTGGT CTAAccccctTAActcaAttccca
NMR 8	JH602050	17076305	17076585	ACACTGACGACATGGTTCTACA aggTtggaTattttaggaagtgtgT	TACGGTAGCAGAGACTTGGT CTAAtattcctaataaAcccaAActttcca
NMR 9	JH602201	46883	47029	ACACTGACGACATGGTTCTACA TtgtaTTTtgTaaTTTTtgTaggtggg	TACGGTAGCAGAGACTTGGT CTAccctAaAcccaacaccctctc
NMR 12	JH602080	19159913	19160063	ACACTGACGACATGGTTCTACA TaggggTaggTtgtgggagg	TACGGTAGCAGAGACTTGGT CTActcactccaccctcaccaac
NMR 13	JH602069	15422990	15423216	ACACTGACGACATGGTTCTACA gttgTTTTagTtgggTaTtgtaggt	TACGGTAGCAGAGACTTGGT CTAActctAcctctAAccaAAccaAcc
NMR 14	JH602048	11061391	11061687	ACACTGACGACATGGTTCTACA aggggTagTtgggTTaggTT	TACGGTAGCAGAGACTTGGT CTAtctcaccacaAtAcccccaA
NMR 15	JH602044	?	?	ACACTGACGACATGGTTCTACA gggaaTTaggagTtggagggg	TACGGTAGCAGAGACTTGGT CTAacacctttcaaaaccaAaatttcct
NMR 16	JH602051	26386554	26386712	ACACTGACGACATGGTTCTACA gagggagttggggtgaatgt	TACGGTAGCAGAGACTTGGT CTAAcccaccaaacatcaaccct

Chr: chromosome/contig. position, Spos: start position, Epos: end position, when mapped to the naked mole rat genome (https://www.ncbi.nlm.nih.gov/pubmed/25172923). NMR15 could not be unambiguously mapped to a position on JH602044. The first part of the primer sequences in upper case are the common adapter sequence for fluidigm C1 amplification. Upper case bases in the target sequences that follow are positions which would be Cs in the reference, but as these primers are used against bisulfite converted DNA, they have been changed to Ts (or As in the case of the reverse primers).

Supplementary Table 3. 23 NMR aDMPs identified from the 51 CpGs with chromosome/contig. position (Chr) and position, when mapped to the naked mole rat genome (https://www.ncbi.nlm.nih.gov/pubmed/25172923).

	Chr	Position
1	JH602136	8746439
2	JH602136	8746449
3	JH602136	8746436
4	JH602136	8746451
5	JH602136	8746445
6	JH602048	1786884
7	JH602048	1786857
8	JH602048	1786864
9	JH602136	8746467
10	JH602080	19159979
11	JH602048	1786873
12	JH602048	1786866
13	JH602136	8746485
14	JH602048	1786879
15	JH602201	46935
16	JH602123	5429210
17	JH602136	8746420
18	JH602080	19160007
19	JH602048	1786845
20	JH602123	5429173
21	JH602048	1786852
22	JH602136	8746480
23	JH602048	1786834

Supplementary File

Please browse Full Text version to see the data of Supplementary File 1.

Supplementary File 1. Raw data set. This file contains data from all the samples for the 51 NMR CpGs expressed as methylation beta values (ratio of methylated and unmethylated alleles expressed between 0 and 1.0).