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Research Article

Decoupling of DNA Methylation Status and Gene Expression Levels in Aging Individuals

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ABSTRACT

Aging is a multi-factorial process, where epigenetic factors play one of the major roles in declines of gene expression and organic function. DNA methylation at CpG islands of promoters can directly change the expression of the neighbouring gene mostly through inhibition. Furthermore, it is known that DNA methylation patterns change during aging. In our study, we investigated gene regulation through DNA methylation of genes up- and downregulated in long-lived people compared to a younger cohort. Our data revealed that comparatively highly methylated genes were associated with high expression in long-lived people (e.g. over 85). Genes with lower levels of methylation were associated with low expression. These findings might contradict the general model used to associate methylation status with expression. Indeed, we found that methylation in the promoter regions of all investigated genes is rather constant across different age groups, meaning that the disparity between methylation and expression only happens in older people. A potential explanation could be the impact of other epigenetic mechanisms, possibly related to stress.

KEYWORDS

Longevity; DNA methylation; gene expression; epigenetics; CpG islands, CpG sites; aging

INTRODUCTION

With aging comes a loss of cognitive abilities, reduction of organ function, and a rapidly increasing risk of chronic diseases such as Alzheimer's type of dementia, diabetes and cancer [1]. Two-thirds of deaths worldwide can be attributed to age-related diseases [2, 3]. Over the course of human evolution, life expectancy has risen dramatically alongside diseases associated with age [4, 5]. Maintaining health in a long-living population can powerfully benefit from understanding the mechanisms that protect older people from these diseases, for example by investigating those who have reached 90 years or older without suffering from major impairments [2, 6]. Despite extensive studies in areas of genetics, transcriptomics and epigenetics, the secret of long-lived healthy human individuals is still unknown [7, 8]. It is, however, widely accepted that this phenotype is a result of a combination of genetic, epigenetic and environmental factors. A number of twin studies have shown that genetic factors make a significant contribution [9–11]. Recently, this has led to large-scale studies attempting to associate genetic variation with longevity [2, 12, 13]. However, so far only three loci significantly associated with human longevity or healthy human aging have been identified, genetic variations on APOE, FOXO3A and 5q3.33 [2, 14–16]. The APOE $\varepsilon 4$ allele, representing a risk factor for Alzheimer's disease, has been found to be less frequent in centenarians compared to younger controls [17]. The Single Nucleotide Polymorphism (SNP) rs2149954 on chromosome 5q3.33 has shown to be associated with lower all-cause mortality in long-lived individuals [14, 18, 19].

More is known about the relationship between gene expression and longevity. A widely accepted assumption in literature is that long-lived people have a better tolerance against stress, (age-related) diseases and other negative influences. The phenomenon is also known as resilience. Certain genes can affect lifespan and the quality of health [20–22]. A hypothesis is that these genes may have a higher expression in such individuals than in those who die at an average age. However, little is still known about the way most of these genes are regulated. One level at which genes can be regulated directly is DNA methylation, an epigenetic mechanism [23] involving the binding of methyl groups predominantly to cytosine bases in DNA. Regions of DNA that are at least 200 kb in length in which adjacent C and G bases make up more than 50 % of the sequence are called CpG islands (CGIs) [6, 24, 25]. DNA methylation in CpG islands of promoter regions is known to impair the expression of the neighbouring gene [25, 26]. Epigenetic features beyond DNA methylation are known to change with age through environmental influences [27]. As the environment has the largest impact on lifespan [9–11], epigenetics may play an important role in aging

and longevity. It is indeed already known that DNA undergoes a global hypomethylation through the whole life span, but site-specific examples of hypermethylation are also found, particularly in CGIs [28–30].

A large-scale eQTL study by Häsler et al. 2017 compared the expression profiles of long-lived individuals (LLIs, aged 90-104 years) and a younger control group (aged 20-55), identifying 6075 differently expressed genes in whole blood samples [31]. In our study, we analyzed the gene expression data from Häsler and colleagues [31] in combination with DNA methylation data from the public database NCBI to gain new insights into the epigenetic regulation of gene expression over the course of aging. The more specific aims were to determine whether and how DNA methylation influences the up- and downregulation of these genes, to uncover differences in DNA methylation between the groups of genes whose expression was affected in opposite ways, and to compare the DNA methylation status of these genes in young (aged 19-45) and very old (aged 85-101) individuals.

DATA

A summary of all data that will be discussed within the following paragraph is provided in Supplementary Information Table S1.

Gene expression data

The raw RNA-seq data investigated in this paper were taken from the study by Häsler et al. (2017) [31]. Häsler and colleagues compared the gene expression profiles of LLIs (aged 90-104, n = 55) to those of younger controls (aged 20-55, n = 73) and identified 6075 genes in whole blood whose levels of expression were significantly different. In our study, we re-analyzed the gene expression analysis of this paper getting all differentially expressed genes of young and old (see Methods). For more descriptive results, we later used the 25 most significant genes whose expression was upregulated and 25 which were downregulated in the LLIs compared to controls of the 6075 genes of the eQTL study; for the full list of these 50 genes, see Table S1 in the study of Häsler et al. (2017).

DNA methylation data

Methylation data were obtained from a study of aging in which methylome profiles were generated from the whole blood of 656 individuals whose ages ranged widely (from 19-101 years) [32]. Methylation detection in the study was carried out using the Illumina Infinium 450k Human DNA methylation Bead chip; the analysis yielded methylation states for more than 450.000 CpG sites spread over the whole genome [33]. The methylation status of a specific CpG site was calculated by the ratio of the number of methylated sites compared to the total combined number of both methylated and unmethylated sites. The values assigned to sites were represented between 0 (unmethylated) and 1 (methylated). The choice of the dataset was based on the wide range of ages of individuals in the study, including long-lived individuals with an age over 85 years (n = 53), which allowed us studying expression methylation correlation from similar age groups. The methylation data used in the study are available in the Gene Expression Omnibus Database of NCBI under accession number GSE40279.

METHODS

Differential expression analysis

The differentially expressed genes (DEG) were determined using R. DESeq2 was used to determine size factors and normalize the data. As our sample size was large and the memory management of regular tools not handy for this kind of dataset, we performed a Wilcoxon rank-sum test on normalized data followed by multiple testing correction using the Hochberg method. The output revealed 3088 DEG; with 1345 up- and 1743 downregulated genes in LLIs. In the following, we will use the definition “all DEG” for the DEG we have calculated on our own (subset 1). The 25 most significant upregulated and the 25 most significant downregulated genes were retrieved from the short-list by Häsler and colleagues [31](subset 2). A further subset (subset 3) containing all genes of the whole genome was used as reference comparison.

Selection of genomic regions, promoters and CpGs

The promoter regions of all three subsets were identified via the publicly available UCSC Genome Website “Table Browser“ [34, 35]. The length of the promoter regions was defined as 2000 bp upstream and 500 bp downstream of the transcription start-site of each gene. The length of promoter regions was also tested in subsequent analysis of DNA methylation and -2000, +500 were selected as reasonable values; see Supplementary Information Figure 1. Subsequently, we searched for CGIs for all filtered promoter regions (using Table Browser from the UCSC website) and the CpG sites within the CGIs were extracted as IDs. 15 of the 25 upregulated genes had at least one CGI in the promoter region, whereas among the 25 downregulated genes 16 genes showed this mark (Table 1). We present a summary of the genes with CGIs in the promoter region for all subsets in the Supplementary Information Table S2.

Statistical analysis

R was used for all statistical analysis steps. To compare the average methylation status of the upregulated and the downregulated genes in each individual of both gene subsets as well as for the whole genome, a t-test was performed, using a significance level of 0.05.

To explore functionality of gene regulation, we then focused on the 25 most up- and downregulated genes. We used ordinary linear regression analysis to investigate the extent of potential relationships between

Table 1: Upregulated and downregulated genes with CGIs in the promoter region; including the regression coefficient, R^2 and p-value for the average methylation status and the age. Coefficient values are grouped into either positive or negative coefficients and sorted in descending order by the amount of the value. Gray-deposited genes do not show a significant coefficient.

Upregulated genes	Coefficient	R^2	p-value
All genes	4.3417E-05	0.02737276	2.055E-05
POLB	-0.00011529	0.02729836	2.11E-05
SLC27A3	-9.90E-05	0.01829921	0.0005126
TRIP4	-6.61E-05	0.01296809	0.0034932
DNAJC1	-3.90E-05	0.01876343	0.0004343
DES	0.00087522	0.16070207	1.02E-26
MANEAL	0.00012929	0.03074176	6.27E-06
PHLDA3	0.00012286	0.04521801	3.82E-08
PAQR4	9.33E-05	0.00965713	0.01179377
HOXB7	5.55E-05	0.03074771	6.25E-06
VAMP5	0.00017412	0.00215643	0.2349325
LINC00899	-1.65E-05	0.000367	0.624
PSMB8	5.12E-06	0.00014719	0.7564415
SAP30	-3.09E-06	5.79E-05	0.84576
RIPK3	3.02E-06	1.49E-05	0.9212891
Downregulated genes	**Coefficient**	**R^2**	**p-value**
All genes	5.6911E-05	0.0409773	1.704E-07
RASGRF2	0.00021243	0.04510329	3.98E-08
NELL2	0.0002086	0.1065761	9.35E-18
SFRP5	0.00016533	0.06150759	1.19E-10
NT5E	0.00014536	0.03180103	4.31E-06
RCAN3	0.00012215	0.05495721	1.22E-09
N4BP3	7.34E-05	0.01189972	0.0051582
CACHD1	7.28E-05	0.01976716	0.0003037
CAMK4	5.73E-05	0.03248401	3.39E-06
ZFYVE9	4.55E-05	0.00746807	0.0268769
NIPAL3	-0.00041598	0.05202189	3.45E-09
AQP3	4.01E-05	0.005764	0.0519
NOG	6.13E-05	0.00451352	0.0855455
SPTBN1	2.80E-05	0.003919	0.1091766
GPRASP1	9.24E-07	0.001188	0.378
CD248	-7.38E-05	0.00118756	0.3782035
SERPINE2	0.00012215	5.61E-06	0.9517

age and the mean methylation status of promoters for both groups of genes. The explanatory variable corresponds to the age and the average methylation status represents the dependent variable. A summary table of the linear regression analysis for all DEG can be found in the Supplementary Information Table S4.

RESULTS

Age-related upregulation of gene expression is associated with a higher methylation status

First, we investigated whether there is a difference in the overall methylation status between the upregulated and the downregulated genes. We compared the average methylation status of both subsets within the promoters with that of the whole genome. The average methylation status of the upregulated genes was significantly higher than the methylation status of the downregulated genes in both gene groups ($mean_{up25}$ = 0.1122 vs. $mean_{down25}$ = 0.0694, $mean_{upall}$ = 0.0814 vs. $mean_{downall}$ = 0.0726, p < 2.2 x 10^{-16} ; Figure 1, Supplementary Information Table S3). The 25 upregulated genes together yield nearly the same mean value for the methylation status as that of the whole genome ($mean_{Genome}$ = 0.1127, Figure 1, Supplementary Information Table S3). A t-test between the genome and the 25 upregulated genes verified the similarity of the average methylation status with p-value = 0.141. In blood cells, our analysis revealed an average methylation status with a value close to 0 for all genes, which indicates an overall low methylation status of CGIs in promoter regions.

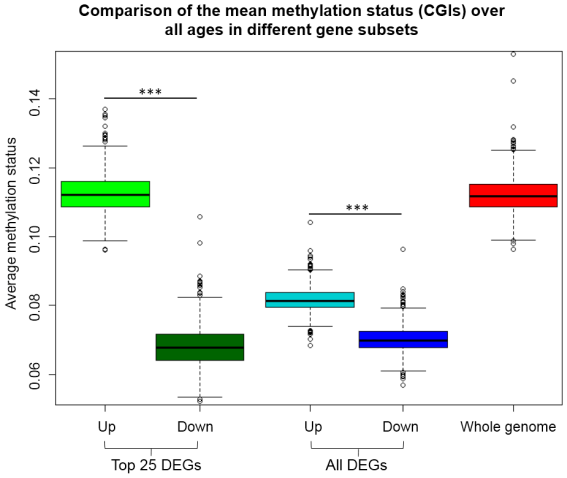

Figure 1: Up- vs. downregulated genes vs. whole genome. Distribution of the average methylation status of promoter-associated CpG sites in CGIs of upregulated and downregulated genes over all age groups in different subsets of genes. Tissue: whole blood. *** p<0.001.

Level of methylation in promoters associated to long-lived people pre-exists at early stages of life

In the following, we investigated a possible correlation between age and mean methylation status within the promoters of the upregulated and downregulated genes and the whole genome over the age. A small but significant increase of the regression coefficient was observed for all different subsets (Figure 2A, Supplementary Information Table S3). The average methylation status of the whole genome revealed the highest coefficient ($coeff_{Genome}$ = 9.18 x 10^{-5} , p-value = 2.34 x 10^{-9}) and the highest R^2-value of nearly 6 % over the age. However, the calculated values are still small. The slope of the 25 upregulated genes was 4.34 x 10^{-5} (p-value = 2.06 x 10^{-5} , significance level = 0.05). The R^2-value was equally significant but relatively small at 2.74 %. Both values are rather small: age accounts for only 2.74 % of the variance of the average methylation status in the investigated promoter regions. The regression coefficient of the 25 genes whose expression was lower in LLIs than in younger individuals was 5.69 x 10^{-5} (p-value = 1.704 x 10^{-7}), associated with a low R^2-value of 0.041. This is about double the value of the upregulated genes. The comparison of the small gene subgroup with that of all DEG showed that the upregulated genes of the respective group revealed both a similar coefficient and a similar R^2-value. The same phenomenon could be found in the downregulated genes. All the slopes are very weak, which, however, strongly suggests that the methylation of these genes changes very little over the course of a lifetime.

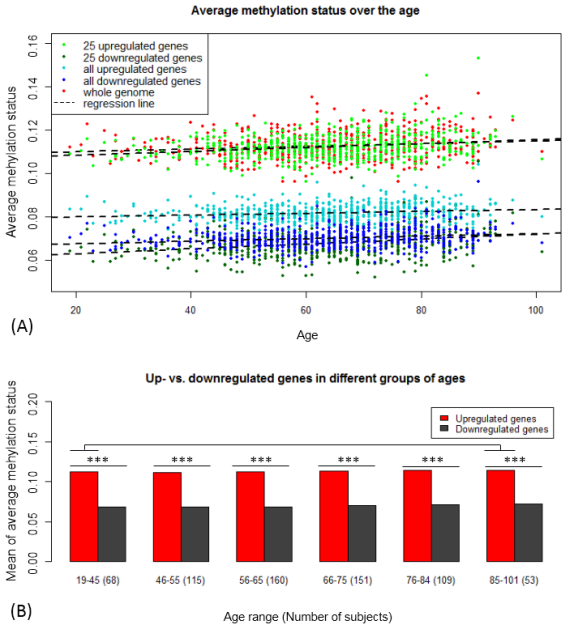

Figure 2: (A) Average methylation status of promoter-associated CpG sites of all different subsets (e.g. legend). The lines show the regression coefficient. **(B)** Means of the average methylation status of the 25 upregulated (red) and the 25 downregulated (grey) genes in six different age-intervals. *** p<0.001.

As we could find the most significant difference between the up- and downregulated genes in the second subset, we used this group of 50 genes for further analysis (Supplementary Table S3). Next, we wanted to determine whether the average methylation of the 50 genes differs significantly when the 656 individuals were clustered by age. Using a paired t-test, a significant difference with p-values < 2.2 x 10^{-16} could be found in all six age groups between the upregulated and the downregulated genes (Figure 2B). In order to be able to carry out a direct comparison of young and old individuals later on the distribution of age groups was chosen to be similar to those from the gene expression analysis in [31]. To have a similar number of subjects in all groups, the age intervals were divided as follows: 19-45 (similar to 20-55 in [31]), 46-55, 56-65, 66-75, 76-84 and 85-101 (similar to 90-104 in [31]). The mean of the differences ranged from 0.042 (age: 85-101) and 0.044 (age: 19-45), whereby means of the upregulated genes were higher in all age intervals. A t-test comparing the average methylation status of the oldest group (85-101 years) with the youngest group (19-45 years) revealed a non significant difference for the upregulated genes (p-value = 0.05826).

Methylation status of most up- or downregulated genes related to LLIs does not change with age

The general model of gene regulation predicts a negative correlation between levels of promoter methylation and expression [36–38]. Results suggest that age tends to be accompanied by site-specific hypermethylation [39]. LLIs exhibit a different pattern in this study: the methylation of promoters is found to not correlate with expression and remains stable over a person's lifetime. This was confirmed by submitting each gene to a linear regression (Table 1). Four of the upregulated genes seem to indicate a slight influence of methylation on gene expression, when the average methylation status across CpGs over age is analysed: POLB (Figure 3A), SLC27A3, TRIP4, and DNAJC1. These significant regression coefficients were negative, in association with age (Table 1). In addition, among the genes with a significant regression coefficient, the proportion of hypomethylated properties (decreasing methylation with increasing age) was larger in the upregulated genes than in downregulated genes (Table 1). This suggests that the expression of such highly expressed genes is partially influenced by the decreasing DNA methylation which occurs with age. All downregulated genes with a significant coefficient exhibited increasing methylation with age, except for one, NIPAL3 (Figure 3C). Otherwise, the change corresponds to their expression profile, since the expression of a gene is more likely to be lower when the CGIs of the promoter are more highly methylated. Nevertheless, in every investigated case, the R^2-value was very low. This represents the degree to which the methylation status can be explained by age. The significant regression coefficient of the genes, however, with a value close to zero corresponds to the model of increasing methylation by age. The low R^2-value indicates that other factors beyond DNA methylation play a role in the regulation of gene expression during aging as well.

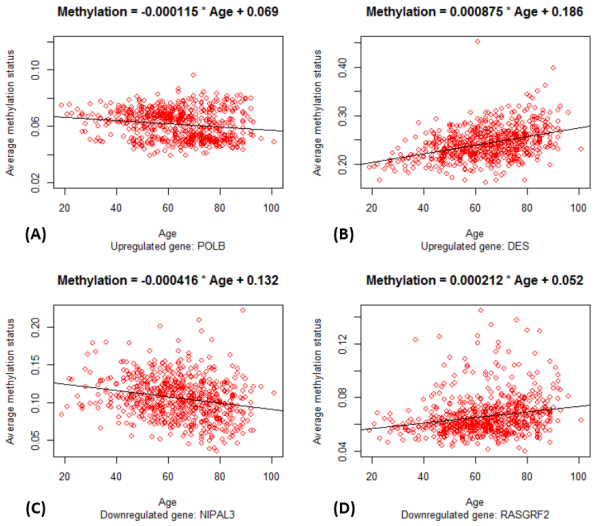

Figure 3: Genes with the highest and lowest slope value. **(A)** shows the lowest coefficient and **(B)** the highest coefficient of the upregulated genes during aging. **(C)** displays the lowest and **(D)** the highest slope of the downregulated genes.

DISCUSSION

In this study we present hints showing that the promoters of genes which are highly expressed in aging people exhibit comparatively high levels of methylation already present in earlier stage of life. Usually, highly methylated CGIs in promoters are assumed to represent gene silencing. Nevertheless, if gene expression in aging people increases or decreases while the methylation status remains more or less the same, this can probably be ascribed to other epigenetic mechanisms that come into play and suppress the normal effects of DNA methylation. At some point over a lifetime, methylation levels no longer suffice to regulate the levels of the expression of certain genes. The role of epigenetic mechanisms participating in this process is currently unclear. As a result, genes can be differentially expressed despite a comparable degree of DNA methylation status, and that the genes already expressed at low levels are impaired by lowering expression even further.

Ideally, data on methylation and expression over the course of aging could be investigated for the same individuals. To our knowledge, however, those data are not available. In the future, it would be very interesting to generate a methylation data set and the respective gene expression profiles in a group of people over 85 years of age, and to compare the results with similar data from 60-80-year-old individuals, which would produce more clear result in specific real differences. Here, we provide a first indication that aging people experience a repression of gene expression regulation through DNA methylation over their lifetimes, which may be the result

of epigenetic mechanisms and other factors beyond DNA methylation in promoter-associated CpG islands.

The next steps should be integrating data regarding the activity of other epigenetic mechanisms to obtain a more comprehensive view of the regulation of genes related to longevity. Other epigenetic mechanisms have been shown to undergo changes that influence gene expression patterns over the course of aging or in relation to longevity [40]. One example is a general loss of histones that comes with age and decreases the stability of the genome [40, 41]. The best strategy to determine how these factors combine to influence genes which are highly expressed in long-lived individuals would be to launch a large QTL study in which many types of epigenetic data, including histone modifications, DNA methylation, histone concentrations and other mechanisms would be considered in parallel in the same subjects. This would provide invaluable data toward understanding how the many changes that cells experience over time interact with each other in promoting health into the last years of our lives.

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AUTHOR CONTRIBUTIONS

AW performed data analysis, interpreted the results and wrote the text. DF interpreted the results, contributed to designing the project, writing and editing the text. HT and SK were involved in the statistical data analysis and in editing the text. SG and KE designed and supervised the project and edited the text. All authors read over and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY DATA

Supplementary Information file listed below, is available at Genomics and Computational Biology online.

Supplementary Information. This file includes supplementary Information tables about the data and a more detailed view on some results.

ABBREVIATIONS

bp: base pairs

CGI: CpG island

CpG: CpG site

DEG: differentially expressed genes

LLIs: long-lived individuals

NCBI: National Center for Biotechnology Information

QTL: quantitative trait loci

SNP: Single Nucleotide Polymorphism

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