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Article

Methylation-wide human study identified age and BMI-independent association of differentially methylated regions and leukocyte telomere length

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Abstract: Background: Telomere length (TL) shortening process is associated with several known environment and individual determinants. DNA methylation is the most studied epigenetic process and may be associated with TL. We investigated the associations between DNA methylation and TL in peripheral blood. Methods: Methylation wide association study was conducted in 47 women (37.1±8.8 years) with different nutritional status. Association between TL and DNA methylation levels were explored by univariate and multiple linear regression models, corrected by age and Body Mass Index. Corrections for multiple comparisons by Benjamini-Hochberg test was also performed. WEBGestalt was used to identify pathways that are responsible for regulating TL. Results: We found negative correlations between TL and BMI (r = -0.641; p = 0.001), abdominal circumference (r = -0.622; p = 0.001) and fat mass (r = -0.656; p = 0.001). 44 CpGs sites were associated with TL, independent of age and BMI. The most of these sites were negatively correlated with TL. For the 7 remained sites, DNA hypomethylation were associated with shorter TL. These CpGs were related to nine different pathways, including thermogenesis, cancer, glutamatergic and serotonergic synapse. Conclusion: There is an epigenetic contribution in TL, independent of nutritional status and age. Genes related to TL are involved in important metabolic pathways.

Keywords: telomere length; DNA methylation; DNA microarray

1. Introduction

Telomeres are DNA nucleoprotein structures located at the end of eukaryotic chromosomes consisting of hundreds to thousands tandem repeats of TTAGGG [1,2], that play an important role in preventing chromosomes from erosion and end-to-end fusion, and thereby maintaining chromosome's integrity [1]. Telomeres are shortened at each cell division and this mechanism decrease chromosomal stability. Also, telomere erosion believed to be accelerated by oxidative stress and inflammation [3,5], which increases risks for chronic diseases, cancer, cardiovascular disease, and overall mortality [6]. In line of this, there is a significant inter-individual variation in telomere length (TL) shortening rates throughout life [7]. TL shortening process is associated with several known environment and individual inherent determinants, including smoking [8], excessive alcohol consumption [9], diet [10], physical activity [11], obesity [12] and chronic life stress [13]. In

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addition, TL is also influenced by genetic variations. Recent genome-wide association studies (GWAS) have identified several polymorphisms associated with TL [14,15]. Furthermore, recent studies in epigenetic research have demonstrated the contribution of DNA methylation levels in TL [16,19]. DNA methylation, one of the main mechanisms of epigenetic modification, consists in addiction of cytosine residues of cytosine-phosphate-guanine dinucleotides (CpGs), which plays an important role in gene expression control [20,21]. Importantly, both TL and DNA methylation process are involved in aging and disease development and global DNA hypomethylation was previously associated with TL in adults [16,22]. Despite the evidence, the epigenetic basis of TL remains largely unknown. Therefore, we carried out this cross-sectional study to explore the DNA methylation profile in relationship with marker of TL in women. The present study, for the first time, tested the hypothesis that TL would be associated with DNA methylation level, independent of nutritional status and age. First, we performed Human Methylation 450 microarray analysis and measured TL in DNA from women patients. Also, we investigated the associations between methylation at CpG sites and TL in peripheral blood DNA.

2. Results

2.1 Phenotypic Characteristics and Telomere length measurements

Phenotypic characteristics and TL of study participants are described in Table 1. We observed that 48.9% of women had overweight or obesity. Also, we found negative correlations between TL and BMI ($r = -0.641$; $p = 0.001$), abdominal circumference ($r = -0.622$; $p = 0.001$) and fat mass ($r = -0.656$; $p = 0.001$).

Table 1. Phenotypic and TL characteristics of study participants (n=47)

Age (years)	37.1±8.8
Weight (kg)	86.5±26.4
BMI (kg/m^2)	39.9±10.7
Abdominal circumference (cm)	100.6±23
Fat free mass (kg)	51.9±10
Fat mass (kg)	33.3±16.7
TL (T/S ratio)	0.93±0.11

BMI: body mass index. TL: telomere length.

2.2 Epigenome wide DNA methylation analysis

After normalization by quality control, the experiment ends up with 410.586 valid probes. After linear regression, 176 CpG sites (located in 154 unique genes) were associated with TL at the conventional pvalue <0.05 and remained associated after adjustment for multiple comparisons (FDR corrected pvalue <0.05) (Supplementary Table).

However, the identified CpGs did not remained associated with TL after adjustment for BMI and age. Thus, we identified 44 sites within 39 different genes that were associated with TL in whole blood DNA, independent of age and BMI (Table 2). The most of these sites were in promoter region (68.1%), island (52.3%) and in chromosome 6 (18.2%).

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Table 2. 44 CpGs sites related to telomere length after adjustment for BMI and age

CpG	Chromosome	MapInfo	Gene symbol	Gene Region	Chromosome Region
Negative association					
cg05445839		27023088	ARID1A	1stExon	Island
cg03421104		5793847			S_Shore
cg18973238	1	64169293			
cg10499166		23345815	KDM1A	TSS200	Island
cg07283098	3	134514086	EPHB1	TSS200	Island
cg15693898		183602556	PARL	1stExon	Island
cg16009381	5	173043599	BOD1	5'UTR	Island
cg01735621		74162051	C6orf150	TSS200	Island
cg00309106		31865663	EHMT2	TSS200	S_Shore
cg01702338		32812571	PSMB8	5'UTR	S_Shore
cg21128553		3163406			Island
cg25760338		52860082	GSTA4	5'UTR;1stExon	S_Shore
cg24203851	6	111136481	CDK19	TSS200	Island
cg26289450		32158233	PBX2	TSS1500	
cg22592140	7	130132419	MEST	5'UTR	Island
cg14298577		100272703	GNB2	5'UTR	Island
cg27338396		26241120	BNIP3L	Body	S_Shore
cg13551243		146052742	ZNF7	TSS200	Island
cg16274098		92053265	TMEM55A	TSS200	
cg23731089	8	141599208	EIF2C2	Body	
cg08446255		26434378	DPYSL2	TSS1500	Island
cg15573846	11	126152723	TIRAP	TSS1500	Island
cg23766360	12	6193688	VWF	Body	
cg02101876	13	40765110			
cg06488135	14	94641122	PPP4R4	Body	Island
cg25198579		67358136	SMAD3	TSS200	Island
cg08370718		49913307	DTWD1;C15orf33	1stExon	Island
cg00036440	15	3507875	NAT15	5'UTR	Island
cg00239353		3115133	IL32	TSS1500	
cg08676730	17	53828263	PCTP	TSS200	N_Shore
cg13893634		48229117	PPP1R9B	TSS1500	S_Shore
cg04349727	19	51457389	KLK5	TSS1500	
cg17064051		3713345	HSPA12B	TSS200	Island
cg16871527	20	20693360	RALGAPA2	TSS200	Island
cg14263118		57463787	GNAS	3'UTR	Island
cg12818493	21	44527599	U2AF1	5'UTR	Island
cg04454272	22	31795531	DRG1	TSS200	Island
Positive association					
cg01919885	4	3365330	RGS12	Body	Island

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cg13941682		702545	PCGF3	5'UTR	S_Shelf
cg03660010		48342263	SLAIN2	TSS1500	N_Shore
cg17745803	6	29631321	MOG	Body	
cg19916067	8	143389706	TSNARE1	Body	
cg12152384	12	47164566	SLC38A4	Body	
cg00989002	16	52225569			

For all 44 identified CpGs, 84.1% (37 CpGs) were negatively correlated with TL. For these sites, lower DNA methylation levels were associated with bigger TL. On other hand, for remained 7 CpGs sites, DNA hypomethylation were associated with shorter TL (Table 2). Enrichment analysis showed that these CpGs were related to nine different pathways (Table 3), including thermogenesis, pathways in cancer, glutamatergic and serotonergic synapse.

Table 3. Enrichment results for CpGs sites related to telomere length

Gene set	Pathway	Size
hsa04926	Relaxin signaling pathway	130
hsa05225	Hepatocellular carcinoma	168
hsa04714	Thermogenesis	229
hsa05032	Morphine addiction	91
hsa04713	Circadian entraiment	96
hsa05200	Pathways in cancer	526
hsa04724	Glutamatergic synapse	114
hsa04726	Serotonergic synapse	115
hsa04611	Platelet activation	123

3. Discussion

As our mainly result, we identified multiple CpG sites in which DNA methylation levels were associated with leukocyte TL independent of nutritional status and age. For the majority of the identified CpGs sites, lower levels of DNA methylation were associated with bigger TL (negative association). These associated genes were involved in different metabolic pathways, such as thermogenesis, circadian entrainment pathway and cancer pathways. In the same way, Buxton et al. 2014 [16] identified 65 CpG sites at which methylation levels associated with leukocyte TL, of these sites, 78% were positively associated. Also, authors evaluating global DNA methylation evidenced that Alu and/or LINE-1 DNA hypomethylation were associated with shorter TL [25]. We evidenced that, when adjusted for age and BMI and age, the number of CpG sites that remained associated with TL decreased. This is the first evidence of the relation between these CpG sites and TL, however the reason of this association remained unknown. We consider two hypotheses: the loci in which these CpG sites are capable induce changes in telomere length or metabolic pathways, which the genes are associated is capable to affect telomere length. It is already known that epigenetic changes near telomeres could influence its length [16; 26]. Telomeric regions do not contain CpG substrates that are susceptible to methylation by DNA methyltransferases (DNMTs), but subtelomeric region has a high number of CpG sites, which can be methylated [27]. However, the 44 CpGs identified in the present analysis were not in subtelomeric regions, and thus, the present hypothesis cannot be confirmed.

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An important pathway related to TL in the preset study was the circadian entrainment pathway. Previously results evidenced circadian rhythm as a mechanism underlying telomere and telomerase activity [28]. The authors suggest that this association has essential clinical impacts, which a possible link between circadian desynchrony and telomere dysfunction [28]. Moreover, pathways in cancer appear as the major pathway related to TL in our results. According to some authors, in cancer development context, telomere shortening may have antagonistic outcomes that involve genome instability and prevention of cancer progression [29, 30]. Interestingly, there is an already known association between the both above pathway. The circadian clock seems to have an important role in multiple physiological processes, homeostasis and, for this reason may control several cancer hallmarks [31]. Despite many evidence about the contribution of different metabolic strategies for energy homeostasis on TL (i.g green tea supplementation; diet; endurance, interval, and resistance training) [12; 32, 33], according to our knowledge, this is the first study showing the direct association between thermogenesis and TL. Here, different explanations may be point out for this association. First, improvement of oxidative stress and inflammation have been associated with better measurements of TL [34,35]. On other hand, it is already known that phosphate is an essential mineral for body energy generation process and recent association has been made between higher phosphate levels and longer TL [36]. Other pathways evidenced in the present study have being described for the first time. The molecular mechanism that relaxin signaling pathway, platelet activation and glutamatergic/ serotonergic synapse pathway is associated to TL need to be further investigate. Like epigenetic clocks, we expect that DNA methylation level may become a useful biomarker for human aging in interventional studies. Considering that epigenetic biomarkers are still in the nascent stage, we will highlight the importance of future prospective study to confirm DNA methylation level as epigenetic biomarkers of TL and possible human disease. Strengths of our study include the wide analysis of DNA methylation by array technology. Limitations include the small sample size and the age range adopted in the inclusion criteria. However, despite the number of included participants, important associations were found.

4. Materials and Methods

Study design and subjects

We performed our methylation wide association study with 47 women (aged between 20 and 60 years old) from an ethnic mixed population, on different nutritional status (according to body mass index – BMI). We did not included patients a history of metabolic diseases such as Cushing syndrome hypo or hyperthyroidism and those in use of antiobesity medications or hormone therapy. Also, men were excluded to avoid the possible biases due to the hormonal influences. For this cross-over study, patients were evaluated only once. Data collection included anthropometrics (weight, height, abdominal circumference, and BMI) and body composition (fat mass and fat free mass) measurements and blood collection for genetic analysis.

DNA extraction

Genomic DNA was extracted from leukocytes using the Master Pure kit (Epicenter, Madison, WI). DNA fragmentation or RNA contamination was analyzed by 1% agarose gel electrophoresis. All samples were stored at -80°C until analyzed.

Measurements of leukocyte telomere length

Analysis for leukocyte telomere length were performed using the method developed by Cawthon et al. [23], with specific modifications [12]. Briefly, Real-time PCR was performed using a 7500 Fast Real Time PCR System (Applied Biosystems). Assay method

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was optimized for use of both telomere (T) and single copy gene (S) amplifications on the same 96-well plate, with reference standard DNA samples on each plate. Analyses were performed in triplicate PCR reactions. Amplification primers for telomeres included TelF: 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′ and TelR: 5′-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′, and for 36B4u: 5′-CCCATTCTATCATCAACGGGTACAA-3′ and 36B4d: 5′-CAGCAAGTGGGAAGGTG-TAATCC-3′. The relative quantification of TL was determined using the telomere to single copy gene ratio (T/S) [2−(ΔCt(telomere) – ΔCt(Single copy gene) = 2−ΔΔCt], following the parameters of Scheinberg et al, 2010 [24]. For 2−ΔΔCt calculation, each sample was normalized to the average T/S ratio of a reference sample, using the standard curve and validation sample as reference.

Methylation assay

Genomic DNA was bisulfate converted immediately before methylation analysis using EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, CA). Methylation chips manufactured by Illumina (Infinium Human Methylation 450 array, Illumina, San Diego, CA) were used for generating methylation data. Beadchips were scanned with the Illumina HiScanSQ system, and image intensities were extracted with the Genome Studio (2011.1) Methylation Module (v1.8.5). Blood samples from each subject were hybridized to the same physical chip to minimize biases. The methylation level was expressed as a beta (β) value that was calculated as the intensity of the methylated channel divided by the total intensity [β = Max (SignalB, 0) / (Max (SignalA, 0) + Max (SignalB, 0) +100]. A threshold for the significant CpG sites based on Δbeta with a minimum value of 5% (value greater than 0.05 or less than -0.05) and p value < 0.001 was applied.

Statistical analysis

Descriptive statistics consisted of mean and standard deviation (SD) values. Shapiro-Wilk test was used to verify the data normality. t-test for independent samples was used for phenotypic variables and TL comparisons between obese and normal weight women. Association between TL and DNA methylation levels were explored by univariate and multiple linear regression models, in which outcome measurement was TL and confounding variables included age and BMI. Corrections for multiple comparisons by Benjamini-Hochberg test was also performed.The significance level used for the tests was set at $p<0.05$. All analyses were performed by using SPSS Statistics 21.0 (SPSS Inc.).

Pathway Enrichment Analysis

WEBGestalt (WEB-based GEne SeT AnaLysis Toolkit) was used to identify pathways that are responsible for regulating TL. For this, the list of genes associated with TL was submitted for statistical overrepresentation testing (Fisher's exact test) in KEGG (Kyoto Encyclopedia of Genes and Genomes). Pathways were considered over-represented where False Discovery Rate (FDR) $p < 0.05$.

5. Conclusions

The results of present study provide novel insights into the epigenetic contribution in telomere length, independent of nutritional status and age. Also, DNA methylation levels of genes involve in important metabolic pathways such as circadian, cancer and thermogenesis are associated with TL.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Raw data for the primary analyses are available upon request from the corresponding and senior author.

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