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Original Article

Specific Differentially Methylated and Expressed Genes in People with Longevity Family History

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Abstract

Background: We attempt to identify specific differentially methylated and expressed genes in people with longevity family history, it will contribute to discover significant features about human longevity.

Methods: A prevalence study was conducted during October 2017 to January 2019 in Bama County of Guangxi, China and individuals were recruited and grouped into longevity family (n=60) and non-longevity family (n=60) to identify differentially methylated genes (DMGs). The expression profile dataset GSE16717 was downloaded from the GEO database in which individuals were divided into 3 groups, namely longevity (n=50), longevity offspring (n=50) and control (n=50) for identifying differentially expressed genes (DEGs). It was considered significantly different when *P* or adjusted $P \leq 0.05$.

Results: In total, 117 longevity-related hypermethylated genes enriched in interleukin secretion/production regulation, chemokine signaling pathway and natural killer cell-mediated cytotoxicity. Another 296 significant key longevity-related DEGs primarily involved in protein binding, nucleus, cytoplasm, T cell receptor signaling pathway and Metabolic pathway, *H19* and *PFKFB4* were found to be both methylated and downregulated in people with longevity family history.

Conclusion: Human longevity-specific genes involve in many immunity regulations and cellular immunity pathways, *H19* and *PFKFB4* show hypermethylated and suppressed status in people with longevity family history and might serve as longevity candidate genes.

Keywords: Differentially methylated genes; Differentially expressed genes; People; Longevity family history

Introduction

Human longevity is the outcome of environment-gene interaction. DNA methylation, one of the epigenetic modifications, is considered as the way by which environmental factors affect the genome (1), it can be transmitted between generations without transforming genome sequences (2), and often alters with environmental, social influences and individual behavior changes (3-5). The methylation profiles of some CpG sites on disease-related genes could decrease or delay diseases occurrence (6, 7), which suggests that DNA methylation could keep people from diseases, allow them to stay in better health status, and further promote human longevity. Moreover, some special methylated sites could determine animal lifespan (8, 9). DNA methylation degree could not only decide the lifespan but also display the aging level, and some key methylation sites of human beings are discovered and applied to predict people's age (10, 11). Thus, the concept of

DNA methylation age (DNAm age), recommended by some researchers, represents the aging degree (12). Centenarians and their offspring maintain in younger DNAm ages, which are younger than their physical ages (13, 14). The methylation patterns of longevity and their offspring differ from their non-longevity counterparts, and they maintain more stable methylation conditions that probably help them reach exceptionally long lives (15). These observations suggest that DNA methylation affects and determines lifespan and contributes to human longevity.

Many longevity-related genes are found to be differentially expressed in the longevity and their offspring (16, 17), it reveals the gene expression profiles of people with longevity family history, like the DNA methylation patterns, also differ from those of the control, and family heredity is a beneficial factor to longevity. Interestingly, gene expression level is controlled and regulated by DNA methylation, and it is suppressed or overexpressed when hyper or hypomethylated in the promoter region (18).

Therefore, we aimed to identify specific genes that are differentially methylated and expressed in people with longevity family history, and this work will contribute to the discovery of genetic and epigenetic features of human longevity.

Materials and Methods

Subjects

Individuals recruited for differentially methylated genes (DMGs) identification

A prevalence study was conducted during October 2017 to January 2019 in Bama County of Guangxi, China, where is famous for longevity people clustering (19), individuals were recruited and grouped into longevity family (n=60, 9 males, 51 females, mean age=98.4±5.9 yr) and non-longevity family (n=60, 11 males, 49 females, mean age=70.3±12.4 yr). The longevity family was based on these criteria: those individuals were born and grew up in Bama County of Guangxi, China, their grandparents, parents, and themselves were 90 yr and older. The non-longevity family met the following criteria: those individuals were born and grew up in the same local as the longevity family, but their grandparents and parents did not reach 90 yr old because of diseases (accident deaths excluded).

Data for differentially expressed genes (DEGs) identification

The expression profile dataset GSE16717(the preprocessed series matrix files) shared by Leiden Longevity Study was downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16717), in which individuals were divided into 3 groups, namely longevity (n=50, males=26, females=24, mean age=93.4 yr), longevity offspring (n=50, males=25, females=25, mean age=60.8 yr) and control (n=50, males=24, females=26, mean age=61.9 yr) (20). The probe sets that showed no correspondence to any gene symbols were removed, when multiple probe sets corresponded to the same gene, average expression value was calculated and considered the gene`s expression value.

Methods

The method for differentially methylated genes (DMGs) identification

In line with Lisa F, et al (21), 2 ml peripheral blood was collected and used for extracting DNA from each individual in Bama County of Guangxi, China. The DNA sample was adjusted to 100ng/μl, in which 5μl was taken to conduct the DNA pooling for the longevity and non-longevity family groups, respectively. Methylated DNA immunoprecipitation (MeDIP) and microarray analysis (Roche NimbleGen) were performed to detect the DNA methylation profiles firmly conducted by the MeDIP-chip protocol. The case and control samples were labeled as Cy5 and Cy3, respectively. Roche-NimbleGen MS200 Scanner was utilized through which the image signals were transferred into digital signals analyzed by NimbleScan ™ 2.6 software. The ratios of Cy5 versus Cy3 signals were calculated, nor-

malized, and transformed to Log2 ratio. A one-sided Kolmogorov–Smirnov (KS) test was used to determine *P*-value and peak score (−log10 *P*-value) based on log2 ratio of each probe. The peak score was generated by interval analysis with a cutoff value of 2. The regions with peak scores ≥ 2 were defined as significantly methylated.

The methods for differentially expressed genes (DEGs) identification

The DEGs between the longevity and control groups and those between longevity and their offspring were identified by GEO2R online (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE16717). The DEGs between the longevity and control differed by age and genetics, whereas those between longevity and their offspring differed by age only as they shared the same family heritability. The two DEG sets were conducted into a Venn diagram. Thus, the complement set in the longevity and control presented the longevity-related DEGs differed by family background or genetics.

Significantly key longevity-related DEGs identification

The longevity-related DEGs were clustered into differently colored modules by weighted gene co-expression network analysis (WGCNA)(22) based on a dissimilarity measure (dissimilarity measure = 1-topological overlap measure (TOM), and TOM ≥ 0.15) (23). The significant modules and genes were determined by the highest module significance (MS) and gene significance (GS) values, respectively. According to the WGCNA manual, a significant absolute GS value indicates a biologically important gene, the MS value denotes the average absolute GS value for all genes in each module, and the MM value shows the correlation between the gene expression profile and module eigengene in each module.

Statistical analysis

An F test was performed to compare age between groups, and a chi-square test was applied to analyze gender distribution. Benjamini–Hochberg's procedure was utilized to adjust the *P*-value in GEO2R, *P* or adjusted *P*≤0.05 indicated a significant difference. A significant methylation site was identified when the absolute peak score ≥ 2. The DEGs in the most significant module were analyzed online by the Data for Annotation Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/summary.jsp) (24) for gene ontology and pathway-enrichment to identify biologically significant genes.

Ethical approval

Individuals from Bama County, Guangxi of China got written informed consent, and study protocols were approved by the Institutional Review Board of Guangxi Medical University. Individuals in dataset GSE16717 got written informed consents and study protocols were approved by Leiden Longevity Study.

Results

Differentially methylated genes

Overall, 117 longevity-related hyper and 1,983 hypomethylated genes (not including those on the sex chromosome) were found in the people of longevity family. The hypermethylation genes were located in 21 chromosomes except for chromosome 13, while the hypomethylation genes were located in 22 chromosomes and DMGs mainly clustered in chromosomes 1, 17, and 19. The top gene annotations (GOs) of longevity-related hypermethylated genes were positive regulation of interleukin-5, interleukin-13 secretions (Table 1). The top GOs of longevity-related hypomethylated genes were mostly collagen-activated tyrosine kinase receptor signaling pathway, regulation of extracellular matrix organization and wound healing, the spread of cells and other biological functions (Table 2). The pathway analysis showed the longevity-related hypermethylated genes were enriched in the cellular immunity pathways of chemokine signaling pathway and natural killer cell-mediated cytotoxi-

city. The hypomethylated genes were mainly enriched in the transduction function pathways of olfactory transduction, neuroactive ligand-receptor interaction, and insulin signaling pathway.

Table 1: Top 10 gene annotations (GOs) of longevity-related hypermethylated genes

Sequence	*Category*	*Term*	*Item*	*Genes*	*P-value*
1	Biological process	GO:2000664	Positive regulation of interleukin-5 secretion	13	4.73e -24
2	Biological process	GO:2000667	Positive regulation of interleukin-13 secretion	13	4.73e -24
3	Biological process	GO:2000553	Positive regulation of T-helper 2 cell cytokine production	13	1.18e -23
4	Biological process	GO:2000662	Regulation of interleukin-5 secretion	13	1.18e -23
5	Biological process	GO:2000665	Regulation of interleukin-13 secretion	13	1.18e -23
6	Biological process	GO:2001181	Positive regulation of interleukin-10 secretion	13	1.18e -23
7	Molecular function	GO:0005245	Voltage-gated calcium channel activity	21	1.88e -22
8	Biological process	GO:0031584	Activation of phospholipase D activity	13	1.88e -22
9	Biological process	GO:2000551	Regulation of T-helper 2 cell cytokine production	13	1.88e -22
10	Cellular component	GO:0005891	Voltage-gated calcium channel complex	21	4.18e -22

Table 2: Top 10 gene annotations of longevity-related hypo methylated genes

Sequence	*Category*	*Term*	*Item*	*Genes*	*P -value*
1	Biological process	GO:0038063	Collagen-activated tyrosine kinase receptor signaling pathway	27	2.57e-24
2	Biological process	GO:0038065	Collagen-activated signaling pathway	27	2.57e-24
3	Biological process	GO:0061302	Smooth muscle cell-matrix adhesion	27	2.57e-24
4	Biological process	GO:0010715	Regulation of extracellular matrix disassembly	29	4.89e-22
5	Biological process	GO:1903053	Regulation of extracellular matrix organization	30	8.12e-22
6	Biological process	GO:0044319	Wound healing and spread of cells	29	2.32e-19
7	Biological process	GO:0090504	Epiboly	29	2.32e-19
8	Biological process	GO:0090505	Epiboly involved in wound healing	29	2.32e-19
9	Biological process	GO:0014909	Smooth muscle cell migration	27	1.50e-18
10	Biological process	GO:0014812	Muscle cell migration	27	2.15e-15

Significantly key longevity-related DEGs

Overall, 1,394 DEGs were observed between the longevity and offspring groups, and another 1,262 were detected between the longevity and control groups (adjusted $P<0.05$), 507 longevity-related DEGs were found in the complement set

of longevity and control groups, which differentially expressed in the people with longevity family history.

A high free-scale topology fit degree was observed (Fig. 1) when the soft threshold was more than 14, which suggested that the 507 longevity-related DEGs met the free-scale topology fit criterion and could be conducted by WGCNA. Longevity-related DEGs were clustered into two modules (turquoise and gray, Fig. 2).

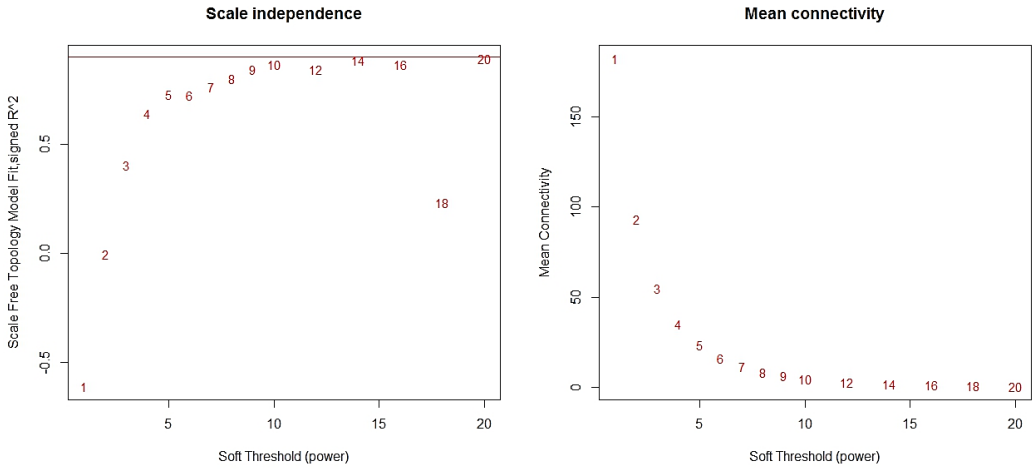

Fig. 1: Free-scale topology model fit analysis for 507 longevity-related DEGs
Note: X-axis represents the soft-threshold power (ß=14) and y axis represents the fit degree (h=0.9)

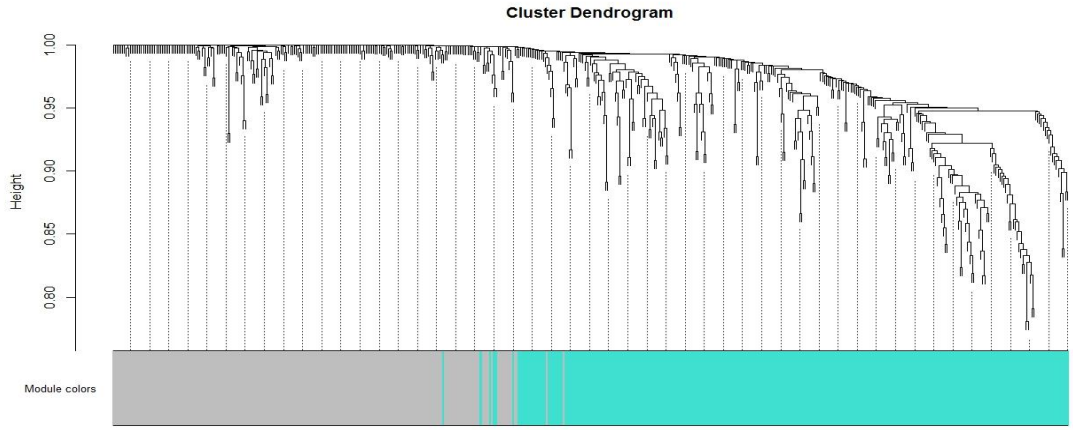

Fig. 2: Gene dendrogram and module colors for 507 longevity-related DEGs
Note: Overall, 507 longevity-related DEGs were clustered to 2 modules (turquoise, grey). The branches in the plot corresponded to the genes and those located at the tips of branches played the highest interconnectedness with the rest of the genes in the module and would be the key genes in the co-expression network

Only the turquoise module (MS = −0.30, $p = 4 \times 10^{-3}$) was significant and contained 296 significant key longevity-related genes. The branches in the plot corresponded to the genes, and those located at the tips of branches played the highest interconnectedness with the rest of the genes and would be the key genes in the co-expression network. The expression profiles of 296 genes were negatively related to lifespan ($r = -0.38$, $P=1 \times 10^{-4}$) and downregulated (for $r<0$) in people with longevity family history. No upregulated longevity-related DEGs were found. The genes in the

grey module failed to be divided into any distinct modules and relatively speaking, might be quite minor to human longevity.

The DAVID analysis shows 296 significant key longevity-related DEGs mainly involved in GO: 0005515~protein binding, GO: 0005634~nucleus, GO: 0005737~cytoplasm, T cell receptor signaling pathway, and hsa01100: Metabolic pathways.

Hyper methylated and down-regulated genes in people with longevity family history

Based on the "hypermethylation suppressing gene expression" theory, the 117 longevity-related hypermethylated genes and 296 significant key longevity-related downregulated genes were integrated, it was found that H*19* and *PFKFB4* were both hypermethylated and downregulated in people with longevity family history (Table 3).

Table 3: The hypermethylated and downregulated genes in people with longevity family history

Gene symbol	*Chromosome location*	*GS value* [a]	*P-value*	*Peak score* [b]
H19	chr11	−0.332	0.020	2.410
PFKFB4	chr3	−0.330	0.021	3.210

[a] GS represents gene significance. [b] represents methylation significance. Peak score ≥ 2 indicates significance

Discussion

Human longevity-related differentially methylated/expressed genes were involved in some immunity regulation functions and cellular immunity pathways

Human longevity is a multi-factorial outcome determined by environmental, epigenetic, and genetic factors. Based on the evidence on DNA methylation modification and its impacts on gene expression, we found some significant differentially methylated/expressed genes and pathways related to human longevity. The longevity-related hypermethylated genes were primarily involved in interleukin secretion/production regulations and cellular immunity pathways. Hypo methylation genes were related to conduction ability, and longevity-related down-regulated genes were involved in the biological function of protein binding and T cell receptor signaling pathway. As receptors and cytokine are usually produced by immunity cells, a strong association is suggested to exist between cellular immunity and longevity. The immunity ability of longevity people is different, and the primary T cell amounts in people from longevity families do not tend to decrease with increasing age (25). In particular, $CD4^+$ T cells increase in long-lived individuals (90 yr and above). Furthermore, T cell receptors show diversity and enable immunity cells to recognize and defend more multiple pathogens (26).

Besides, lymphocyte proliferative capacity, phagocytic ability, and NK cell activity in extremely old people (particularly in the centenarians) could maintain in better conditions (27), and research shows excellent T cell immunity can extend the five-year survival of centenarians (28). These findings indicate cellular immunity, especially T cell immunity, helps people with longevity family history to maintain their healthy status and thereby reach longer lifespans.

Methylated and repressed H19 contributed to longevity probably by keeping people from suffering age-related diseases

H19 is an imprinting gene located in chromosome 11 and encodes a long non-coding RNA *H19*; the latter functions as an oncogene by binding and regulating target genes (29). Moreover, *H19* is highly expressed in various cancers (30, 31) and is upregulated in patients with age-dependent coronary artery disease (32), cataract of the elderly (33), and diabetes by regulating the *IGF2/H19* allele locus (34). *H19* is a disease-susceptible gene when abnormally overexpressed. We found that *H19* was hypermethylated and repressed in people with longevity family history, and the methylated and repressed *H19* status helps people avoid deadly age-related diseases, thereby increasing the

possibility of living longer. Notably, a monozygotic twins–based study shows that the *H19* methylation status is primarily determined by heritable (such as SNPs) but not environmental factors (35), which indicates that the *H19* methylation profile is unique and conservative in people with longevity family history and shows a family-specific feature comparing to the ordinary populations.

Methylated and repressed PFKFB4 might help people to avoid age-dependent malignant carcinomas and get longer lifespan

Glycolysis is the primary energy source of many malignant tumor cells (36), and it depends on a key regulatory enzyme of glucose metabolism encoded by *PFKFB4*, which is always overexpressed in tumors and required for tumor survival (37). In vitro, the *PFKFB4* inhibitor was applied to suppress tumor cell proliferation and growth by blocking glycolysis metabolism (38), which means that tumor cells would be restrained because of glucose restriction when *PFKFB4* is repressed. In our study, *PFKFB4* has shown similar status with *H19* in people with longevity family history, which suggests that the suppressed *PFKFB4* is a protective factor for longevity, and it might keep people away from age-dependent malignant carcinomas. As reported, aging is the risk factor of several cancers and diseases, and many age- and tumor-related genes were suppressed by hypermethylation in longevity and their offspring, thereby preventing the occurrence of these conditions and promoting longer lifespan. Given these findings, the disease-related genes *H19* and *PFKFB4* are speculated to serve as potential candidate longevity genes, as they are always methylated and repressed in those people with longevity family history.

Conclusion

Human longevity is regulated by specific genes that are involved in immunity regulation functions and cellular immunity pathways. Specifically, H19 and PFKFB4 always show hypermethylated and suppressed status in people with longevity family history, they might serve as longevity candidate genes. Further experimental work is needed to confirm the mechanisms about how the candidate genes regulate and promote human lifespan.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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