The Journal of Veterinary Medical Science

NOTE

Internal Medicine

Analysis of DNA methylation of potential age-related methylation sites in canine peripheral blood leukocytes

Genta ITO[1)], Kuniko YOSHIMURA[1)] and Yasuyuki MOMOI[1)]*

[1)]Laboratory of Veterinary Diagnostic Imaging, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

J. Vet. Med. Sci.
79(4): 745–750, 2017
doi: 10.1292/jvms.16-0341

Received: 29 June 2016
Accepted: 22 February 2017
Published online in J-STAGE: 4 March 2017

ABSTRACT. Reliable methodology for predicting the age of mature dogs is currently unavailable. In this study, amplicon sequencing of 50 blood samples obtained from diseased dogs was used to measure methylation in seven DNA regions. Significant correlations between methylation level and age were identified in four of the seven regions. These four regions were then tested in samples from 31 healthy toy poodles, and correlations were detected in two regions. The age of another 11 dogs was predicted using data from the diseased dogs and the healthy poodles. The mean difference between the actual and calculated ages was 34.3 and 23.1 months, respectively. Further research is needed to identify additional sites of age-related methylation and allow accurate age prediction in dogs.

KEY WORDS: age, dog, epigenetics

The ability to accurately predict the age of an adult dog would provide useful information for those involved in veterinary medicine and animal science. For example, companion animal veterinarians take age into consideration when making diagnoses and determining treatment plans. In animal shelters, most people hope to know the age of the animal before they adopt. However, the age of a dog is not always self-evident in situations where breeding is uncontrolled. Several molecular biology-based age-prediction methods have been reported in humans; these include quantitative analysis of T-cell receptor excision circles [4, 19, 21, 32], mitochondrial DNA fragment deletion [1, 2, 11, 15, 20], telomere DNA fragment shortening [24, 28] and age-related DNA methylation levels at specific genomic loci [3, 18, 26, 29, 31]. Although radiography can be used to predict the age of young dogs (less than 1 year old) by their skeletal development [14, 25], the age of adult dogs is usually predicted by subjective observations of characteristics, such as overall appearance or tooth abrasion. Previously, we attempted molecular biology-based age-prediction by measuring the level of blood signal joint T-cell receptor excision circles (sjTREC) in dogs [12]; sjTREC levels reflect the number of T-cells freshly recruited from the thymus and thus potentially indicate age-related thymic atrophy. However, we found no significant correlation between sjTREC levels and age, probably owing to the process of thymic involution that occurs at an early stage of life in dogs. Although age-related telomere length shortening in normal mammary gland tissue has been reported [30], age prediction from the measurement of telomere length has not been attempted in dogs.

DNA methylation is characterized by the addition of a methyl group to a cytosine nucleotide primarily at cytosine-phosphate-guanine (CpG) sites. Short DNA elements that have a much higher density of CpG sites, so-called CpG islands, are often located near transcription start sites. Hypermethylation of these regions is usually associated with transcriptional silencing. With increasing age, some gene-specific CpG dinucleotides can become hypermethylated or hypomethylated [10]. These age-related methylation changes have been used as a biological marker for forensic age-prediction in humans and may have the potential to be used for predicting age in dogs. Recently, several studies have tried to use methylation-predicted age in humans as an indicator of risk of age-related diseases and mortality [9, 10]. In humans, several CpG sites have been identified as age-related markers [3, 7, 13], and quantitative analysis of the methylation levels of multiple genomic regions simultaneously makes age-prediction practical with an average accuracy of 4 to 10 years [3, 18, 26, 29, 31]. The aim of the present study was to identify age-related methylation sites in dogs. We selected loci for testing based on the findings of human studies [5, 6].

In the first experiment, blood samples were obtained from 50 dogs; forty-five of these were client-owned, diseased dogs that were brought to the Kagoshima University Veterinary Teaching Hospital for veterinary care, and five were healthy dogs owned by faculty staff. Genomic DNA was extracted from 100 to 200 μl of EDTA-K2-treated blood using a DNeasy Blood & Tissue Kit (QIAGEN, Venlo, The Netherlands). The eluted DNA solution was concentrated using a DNA Clean & Concentrator-5 Kit (Zymo

*Correspondence to: Momoi, Y., Laboratory of Veterinary Diagnostic Imaging, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan. e-mail: momoi@agri.kagoshima-u.ac.jp

Table 1. Nucleotide sequences of PCR primers designed against bisulfite-treated dog genomic DNA

Primer name	Gene specific primer nucleotide sequence (5′-3′)	Dog chromosome (Chr), NCBI reference ID, Target region, PCR product size (bp)[a)]	Number of CpG sites in the target region	Adjacent gene and remarks	Corresponding age-related CpG site in human (ref.)
A-sense	ttttgggagttttggtgagaa	Chr 5, NC_006587.3, 67230026–67230305, 280	15	GSE1	cg07082267 [6]
A-reverse	ataaaaaaaaccccataaatct				
C-sense	gttttttatgaatgaatattga	Chr 6, NC_006588.3, 8701204–8701368,165	17	VGF	cg21186299 [6]
C-reverse	aataaattaaactcaactaaatc				
E-sense	ggtaaggagaggaggtagttttagg	Chr 24, NC_006606.3, 33293646–33294073, 428	31	SLC12A5	cg07547549 [6]
E-reverse	ccccacctttcaactaaaaatct				
F-sense	ggtgtttaaagtaaattagagagt	Chr 35, NC_006617.3, 23602291–23602560, 270	21	SCGN	cg06493994 [5, 6]
F-reverse	tccaaatcctttcaaaaaaacta				
H-sense	gttaaattttgtttaatttgttgtg	Chr 10, NC_006592.3, 49672242–49672424, 183	13	KCNK12	cg27320127 [5]
H-reverse	aaatcctttcccccaaaaaaacc				
I-sense	ggtttttattattaaggatttttttt	Chr 3, NC_006585.3, 37548040–37548332, 293	13	OTUD7A (3′UTR)	cg01763090 [6]
I-reverse	aaactacaaatttctttatttctcttatta				
J-sense	ggagtttaataggggagagagattt	Chr 5, NC_006587.3, 32051030–32051450, 421	36	BCL6B	cg10137837 [6]
J-reverse	taaaacccctccaaaatacctaac				
K-sense	ttatatagtggggagaaaggtaagtt	Chr 15, NC_006597.3, 45028967–45029372, 406	32	POU4F2	cg05991454 [6]
K-reverse	accctaaaactaaacactaaaatcc				
	Adapter nucleotide sequence (5′-3′)			Remarks	
Sense[b)]	acactctttccctacacgacgctcttccgatct-(Primer)			Adapter for sense primer	
Reverse	gtgactggagttcagacgtgtgctcttccgatct-(Primer)			Adapter for reverse primer	

a) PCR product size was predicted from the gene-specific DNA fragment (excluding adapter nucleotides). b) Adapter nucleotides were added to the 5′ end of each gene-specific primer.

Research, Irvine, CA, U.S.A.) if necessary. Bisulfite treatment of DNA was performed using an EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions, and treated DNA was used as a template for PCR. The genomic region of interest was selected to analyze the methylation levels of CpG regions that correlated to the regions showing age-related methylation changes in humans [5, 6]. Briefly, approximately 1,000 bases flanking previously reported age-related methylation sites were retrieved from the human genome assembly hg38 using the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgGateway). Homologous regions in the canine genome were identified using the Basic Local Alignment Search Tool (BLAST) from the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Homologous DNA regions in the canine genome were identified for some, but not all, of the human age-related regions. In total, 11 genomic regions were identified for analysis in the canine genome. Oligonucleotide PCR primers were designed for bisulfite-treated DNA (converted DNA) using the Methprimer web tool (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) and were synthesized commercially (FASMAC, Atsugi, Japan). The nucleotide sequences of these primers, and their locations within the canine reference genome, are shown in Table 1. Adapter nucleotides were added to the 5′ end of these PCR primers to provide primer binding sites for second-round PCR. First-round PCR was performed using the Epitaq HS PCR Kit (Takara, Kusatsu, Japan) according to the manufacturer's instructions. Second-round PCR was performed using the GoTaq Hot Start Colorless Master Mix (Promega, Madison, WI, U.S.A.) to add a nucleotide adapter for next-generation sequence analysis and nucleotide tags for individual discrimination from mixed samples. PCR products were electrophoresed and extracted from agarose gels, and then purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany).

We failed to amplify DNA fragments from four of the 11 regions. The PCR products from the seven successfully amplified genomic regions were submitted either for 250- or 300-bp pair-end Miseq analysis (Illumina, San Diego, CA, U.S.A.) at FASMAC Co., Ltd., depending on the length of the amplicon. Sequence data from Miseq analysis were processed and then aligned using U-gene software (Unipro, Novosibirsk, Russia) to find specific, highly methylated CpG sites. The numbers of changed (unmethylated) and unchanged (methylated) CpGs at each site were counted individually using a textedit word processor (Apple, Cupertino, CA, U.S.A.). Only regions with more than 200 sequences per individual were included for further analysis. The methylation rate was calculated as the number of unchanged CpGs divided by the sum of changed and unchanged CpGs for each site. Pearson's correlation coefficients were calculated to assess the correlation between each CpG site and the age of the dog. The CpG site with the highest correlation coefficient was selected for each DNA region (Table 2). A scatter plot of the methylation rate at each CpG site against age, with a straight-line approximation, is shown in Fig.1. There was a significant correlation between methylation levels and age at four of the seven CpG sites ($P<0.05$, Pearson's correlation coefficient). Neighboring CpG sites in the same DNA region with the four sites showed 90% (37/41 sites) agreement in their slope direction with age as reported in human cases [5].

We then examined the methylation levels at the four CpG sites with significant correlations between age and methylation in clinically healthy dogs. Blood samples were obtained from 31 toy poodles at the Nishi Animal Hospital (Kagoshima, Japan) or at

Table 2. Correlation between age and methylation level in CpG sites in 50 dogs

Region name	NCBI reference ID	CpG position in reference	Adjacent gene	Correlation coefficient (r)[a)]	*P*-value
A	NC_006587.3	67,230,027	GSE1	–0.367	0.009
C	NC_006588.3	8,701,563	VGF	–0.195	0.175
F	NC_006617.3	23,602,365	SCGN	0.291	0.040
H	NC_006592.3	49,672,518	KCNK12	0.150	0.297
I	NC_006585.3	37,548,075	OTUD7A	0.194	0.196
J	NC_006587.3	32,051,190	BCL6B	–0.354	0.013
K	NC_006597.3	45,029,058	POU4F2	0.421	0.003

a) Pearson's correlation coefficient.

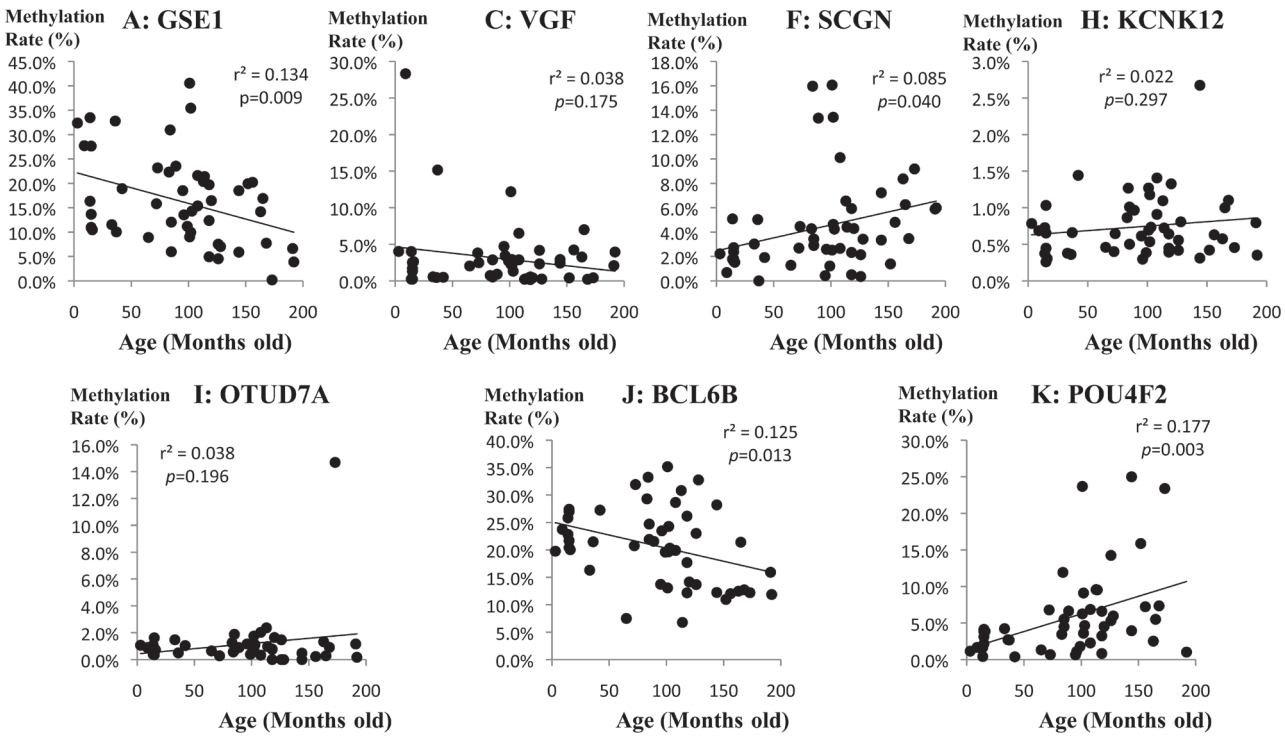

Fig. 1. Scatter plots of age (months) and methylation rates of CpG sites with the highest correlation coefficients in seven DNA regions (A, C, F, H, I, J and K) in the canine genome. These seven regions were expected to show age-related methylation changes based on human studies [5, 6]. The name of the adjacent gene in the canine genome is given as the header label for each scatter plot. Straight-line approximations have been applied to each scatter plot, and Pearson's correlation coefficients are shown.

the Harada-gakuen Animal School (Kagoshima, Japan). Methylation levels were measured as described for the previous samples. There were significant correlations between methylation level and age at two of the four CpG sites that were analyzed (Fig.2). We then attempted age prediction using another 11 client-owned dogs brought to Kagoshima University for veterinary care. Age was predicted by multiple regression analysis of the four CpG sites from the initial 50 client-owned dogs or the 2 CpG sites from the 31 healthy toy poodles (Table 3). The mean absolute difference between actual and predicted age, calculated using multiple regression of data from four CpG sites, was 34.3 months. In five of the 11 dogs, the difference between actual and predicted age was less than 24 months. The mean absolute difference between actual and predicted age calculated using data from two CpG sites was 23.1 months. The difference between actual and predicted age was less than 24 months in seven of the 11 dogs. The maximum difference between actual and predicted age was observed in a 140 month-old dog whose predicted age based on methylation of two CpG sites was 70.8 months. Marked diremption was observed in some dogs. Factors (e.g. nutritional state, breed and disease) that influence methylation levels should be identified. Further exhaustive research, such as genome-wide methylation sequence analysis or development of a cost effective methylation array assay, may identify other, more accurate, age-related changes in the methylation of the canine genome. Consolidation of public databases of canine genome methylation may also help to identify other age-related methylation changes, as has been shown in humans [8].

The biological relevance of some of the genes adjacent to the CpG sites that we studied has been reported, while the function of others, such as Genetic suppressor element 1 (GSE1), is largely unknown. Secretagogin (SCGN) is a calcium binding protein. A

Table 3. Age prediction by multiple regression analysis in 11 dogs

Case No.	Actual age (months)	Predicted age from 4 CpG sites (months)[a)]	Difference (months)	Predicted age from 2 CpG sites (months)[b)]	Difference (months)
1	140	50.3	–89.7	70.8	–69.2
2	50	55.4	5.4	86.8	36.8
3	130	26.1	–103.9	118.8	–11.2
4	15	18.4	3.4	45.3	30.3
5	115	73.7	–41.3	129.7	14.7
6	124	106.4	–17.6	121.4	–2.6
7	120	77.6	–42.4	96.0	–24.0
8	78	86.2	8.2	97.0	19.0
9	110	82.0	–28.0	90.3	–19.7
10	125	93.7	–31.3	106.2	–18.8
11	75	69.0	–6.0	82.3	7.3
Mean absolute Difference			34.3		23.1
Standard deviation (SD)			37.2		30.0

a) Age was predicted by multiple regression analysis using methylation data at 4 CpG sites from 50 dogs (mostly diseased dogs). b) Age was predicted by multiple regression analysis using methylation data at 2 CpG sites from 31 clinically healthy toy.

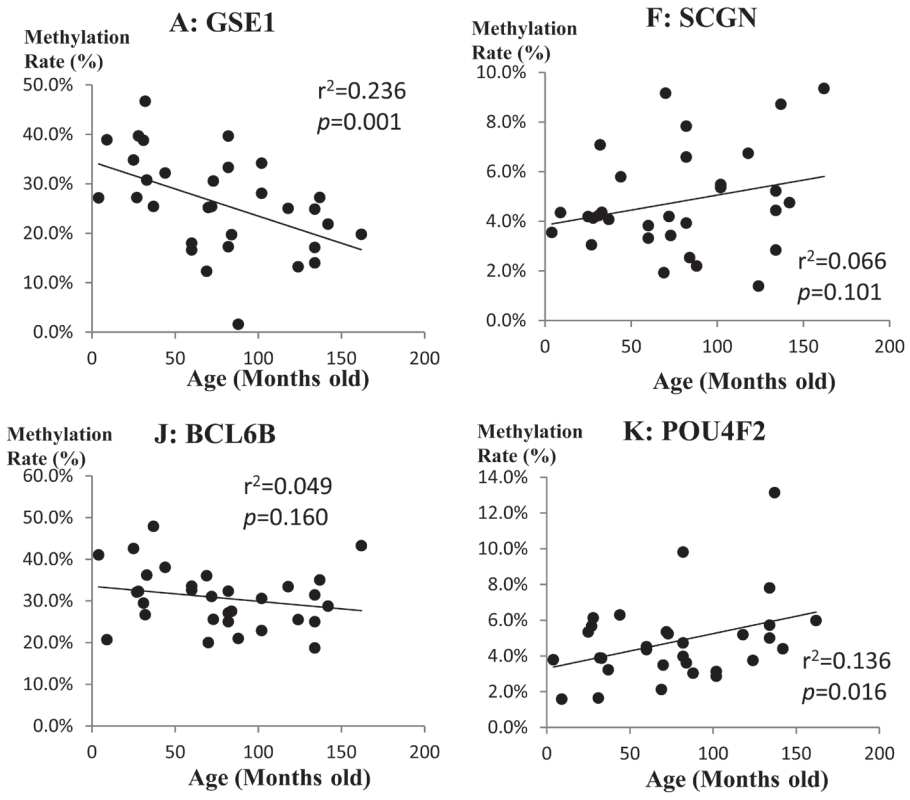

Fig. 2. Scatter plots of age (months) and methylation rates of CpG sites in four DNA regions (A, F, J and K) in blood samples obtained from 31 clinically healthy toy poodles. The name of the adjacent gene in the canine genome is given as the header label. Straight-line approximations were applied to each scatter plot, and Pearson's correlation coefficients (r) and *P* values are shown.

negative correlation between SCGN mRNA expression in peripheral blood mononuclear cells and age has been reported [27]; this age-related decrease in mRNA expression is possibly regulated by epigenetic changes. B-cell CLL/lymphoma 6 member B protein (BCL6B) is a transcription repressor and a potential tumor suppressor. Epigenetic silencing of BCL6B has been reported in human hepatocellular carcinoma [16]. Although expression of BCL6B by CD8 positive T cells has been reported [17], its association with aging is unknown. Interestingly, the correlation between age and methylation observed in dogs at the BCL6B CpG site was the inverse of that found in humans [6]. The reason for this is unclear, and age-related BCL6B expression in dogs needs a further study. POU domain, class 4, transcription factor 2 (POU4F2) is a transcription factor, and hypermethylation of POU4F2 CpG sites

has been reported in some tumors [22, 23], but its biological relevance in aging has not been reported.

Age-prediction by DNA methylation levels has been reported in humans using various tissue samples, including saliva, teeth and brain [2, 5, 10, 26]. However, blood sample are used in most studies, because they are easily obtained. The leukocyte subtype composition of the blood sample does not seem to affect the accuracy of the age prediction [10]. Thus, in this study, we examined methylation levels in dogs using peripheral blood samples. A major limitation of this study is the potential bias in the samples used in the first experiment, which were obtained mostly from sick dogs. Poor health status, especially in the case of age-related diseases, may cause methylation patterns that mimic age-related changes in methylation. A recent study in humans found that some age-related methylation changes become insignificant after restricting the study sample to those without history of major age-related diseases, such as diabetes mellitus, cardiovascular disease, stroke and cancer [6]. Our first experiment included dogs with age-related diseases, such as diabetes mellitus (n=4), cardiovascular disease (n=5) and tumors (n=12). Although the four age-related CpG sites identified in the first analysis were homologous to regions showing age-related changes in humans without age-related diseases [6], the correlations between methylation and age in two of the four sites were not significant in our second experiment using only healthy toy poodles. Ideal age markers should be minimally affected by environmental or genetic factors.

In conclusion, we measured the methylation levels at selected CpG sites in dogs using next-generation amplicon sequencing and identified some age-related changes in methylation. Although age predictions made using the methylation levels at these CpG sites are not yet sufficiently accurate for practical use, further research to identify other age-related methylation sites may make accurate age prediction possible.

ACKNOWLEDGMENTS. The authors are grateful to Drs. Hiroshi Nishi (Nishi Animal Hospital, Kagoshima, Japan) and Takako Nagayoshi (Harada-gakuen, Kagoshima, Japan) for providing the blood samples.

REFERENCES

1. Baumer, A., Zhang, C., Linnane, A. W. and Nagley, P. 1994. Age-related human mtDNA deletions: a heterogeneous set of deletions arising at a single pair of directly repeated sequences. *Am. J. Hum. Genet.* **54**: 618–630. [Medline]
2. Bekaert, B., Kamalandua, A., Zapico, S. C., Van de Voorde, W. and Decorte, R. 2015. Improved age determination of blood and teeth samples using a selected set of DNA methylation markers. *Epigenetics* **10**: 922–930. [Medline] [CrossRef]
3. Bocklandt, S., Lin, W., Sehl, M. E., Sánchez, F. J., Sinsheimer, J. S., Horvath, S. and Vilain, E. 2011. Epigenetic predictor of age. *PLoS ONE* **6**: e14821. [Medline] [CrossRef]
4. Cho, S., Ge, J., Seo, S. B., Kim, K., Lee, H. Y. and Lee, S. D. 2014. Age estimation via quantification of signal-joint T cell receptor excision circles in Koreans. *Leg. Med. (Tokyo)* **16**: 135–138. [Medline] [CrossRef]
5. Day, K., Waite, L. L., Thalacker-Mercer, A., West, A., Bamman, M. M., Brooks, J. D., Myers, R. M. and Absher, D. 2013. Differential DNA methylation with age displays both common and dynamic features across human tissues that are influenced by CpG landscape. *Genome Biol.* **14**: R102. [Medline] [CrossRef]
6. Florath, I., Butterbach, K., Müller, H., Bewerunge-Hudler, M. and Brenner, H. 2014. Cross-sectional and longitudinal changes in DNA methylation with age: an epigenome-wide analysis revealing over 60 novel age-associated CpG sites. *Hum. Mol. Genet.* **23**: 1186–1201. [Medline] [CrossRef]
7. Fraga, M. F., Ballestar, E., Paz, M. F., Ropero, S., Setien, F., Ballestar, M. L., Heine-Suñer, D., Cigudosa, J. C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T. D., Wu, Y. Z., Plass, C. and Esteller, M. 2005. Epigenetic differences arise during the lifetime of monozygotic twins. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 10604–10609. [Medline] [CrossRef]
8. Freire-Aradas, A., Phillips, C., Mosquera-Miguel, A., Girón-Santamaría, L., Gómez-Tato, A., Casares de Cal, M., Álvarez-Dios, J., Ansede-Bermejo, J., Torres-Español, M., Schneider, P. M., Pośpiech, E., Branicki, W., Carracedo, Á. and Lareu, M. V. 2016. Development of a methylation marker set for forensic age estimation using analysis of public methylation data and the Agena Bioscience EpiTYPER system. *Forensic Sci. Int. Genet.* **24**: 65–74. [Medline] [CrossRef]
9. Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S., Klotzle, B., Bibikova, M., Fan, J. B., Gao, Y., Deconde, R., Chen, M., Rajapakse, I., Friend, S., Ideker, T. and Zhang, K. 2013. Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol. Cell* **49**: 359–367. [Medline] [CrossRef]
10. Horvath, S. 2013. DNA methylation age of human tissues and cell types. *Genome Biol.* **14**: R115. [Medline] [CrossRef]
11. Hsieh, R. H., Hou, J. H., Hsu, H. S. and Wei, Y. H. 1994. Age-dependent respiratory function decline and DNA deletions in human muscle mitochondria. *Biochem. Mol. Biol. Int.* **32**: 1009–1022. [Medline]
12. Ito, G., Yoshimura, K. and Momoi, Y. 2015. Gene analysis of signal-joint T cell receptor excision circles and their relationship to age in dogs. *Vet. Immunol. Immunopathol.* **166**: 1–7. [Medline] [CrossRef]
13. Koch, C. M. and Wagner, W. 2011. Epigenetic-aging-signature to determine age in different tissues. *Aging (Albany, N.Y.)* **3**: 1018–1027. [Medline]
14. Koscinczuk, P., Beneventano, A. and Coppo, J. A. 1989. Radiological and biochemical evaluation of bone growth in young dogs. *Acta Physiol. Pharmacol. Latinoam.* **39**: 289–297. [Medline]
15. Lee, H. C., Pang, C. Y., Hsu, H. S. and Wei, Y. H. 1994. Differential accumulations of 4,977 bp deletion in mitochondrial DNA of various tissues in human ageing. *Biochim. Biophys. Acta* **1226**: 37–43. [Medline] [CrossRef]
16. Li, X., Yu, J., Brock, M. V., Tao, Q., Herman, J. G., Liang, P. and Guo, M. 2015. Epigenetic silencing of BCL6B inactivates p53 signaling and causes human hepatocellular carcinoma cell resist to 5-FU. *Oncotarget* **6**: 11547–11560. [Medline] [CrossRef]
17. Manders, P. M., Hunter, P. J., Telaranta, A. I., Carr, J. M., Marshall, J. L., Carrasco, M., Murakami, Y., Palmowski, M. J., Cerundolo, V., Kaech, S. M., Ahmed, R. and Fearon, D. T. 2005. BCL6b mediates the enhanced magnitude of the secondary response of memory CD8+ T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 7418–7425. [Medline] [CrossRef]
18. Marioni, R. E., Shah, S., McRae, A. F., Chen, B. H., Colicino, E., Harris, S. E., Gibson, J., Henders, A. K., Redmond, P., Cox, S. R., Pattie, A., Corley, J., Murphy, L., Martin, N. G., Montgomery, G. W., Feinberg, A. P., Fallin, M. D., Multhaup, M. L., Jaffe, A. E., Joehanes, R., Schwartz, J., Just, A. C., Lunetta, K. L., Murabito, J. M., Starr, J. M., Horvath, S., Baccarelli, A. A., Levy, D., Visscher, P. M., Wray, N. R. and Deary, I. J. 2015. DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol.* **16**: 25. [Medline] [CrossRef]

19. Meissner, C., von Wurmb, N. and Oehmichen, M. 1997. Detection of the age-dependent 4977 bp deletion of mitochondrial DNA. A pilot study. *Int. J. Legal Med.* **110**: 288–291. [Medline] [CrossRef]
20. Ou, X. L., Gao, J., Wang, H., Wang, H. S., Lu, H. L. and Sun, H. Y. 2012. Predicting human age with bloodstains by sjTREC quantification. *PLoS ONE* **7**: e42412. [Medline] [CrossRef]
21. Ou, X., Zhao, H., Sun, H., Yang, Z., Xie, B., Shi, Y. and Wu, X. 2011. Detection and quantification of the age-related sjTREC decline in human peripheral blood. *Int. J. Legal Med.* **125**: 603–608. [Medline] [CrossRef]
22. Pradhan, M. P., Desai, A. and Palakal, M. J. 2013. Systems biology approach to stage-wise characterization of epigenetic genes in lung adenocarcinoma. *BMC Syst. Biol.* **7**: 141. [Medline] [CrossRef]
23. Reinert, T., Modin, C., Castano, F. M., Lamy, P., Wojdacz, T. K., Hansen, L. L., Wiuf, C., Borre, M., Dyrskjøt, L. and Orntoft, T. F. 2011. Comprehensive genome methylation analysis in bladder cancer: identification and validation of novel methylated genes and application of these as urinary tumor markers. *Clin. Cancer Res.* **17**: 5582–5592. [Medline] [CrossRef]
24. Ren, F., Li, C., Xi, H., Wen, Y. and Huang, K. 2009. Estimation of human age according to telomere shortening in peripheral blood leukocytes of Tibetan. *Am. J. Forensic Med. Pathol.* **30**: 252–255. [Medline] [CrossRef]
25. Seoudi, K. 1947. X-ray examination of epiphyseal union as an aid to the estimation of the age in dogs. *Br. Vet. J.* **103**: 150–155. [Medline]
26. Soares Bispo Santos Silva, D., Antunes, J., Balamurugan, K., Duncan, G., Sampaio Alho, C. and McCord, B. 2015. Evaluation of DNA methylation markers and their potential to predict human aging. *Electrophoresis* **36**: 1775–1780. [Medline] [CrossRef]
27. Tan, W. S., Lee, J. J., Satish, R. L. and Ang, E. T. 2012. Detectability of secretagogin in human erythrocytes. *Neurosci. Lett.* **526**: 59–62. [Medline] [CrossRef]
28. Tsuji, A., Ishiko, A., Takasaki, T. and Ikeda, N. 2002. Estimating age of humans based on telomere shortening. *Forensic Sci. Int.* **126**: 197–199. [Medline] [CrossRef]
29. Weidner, C. I., Lin, Q., Koch, C. M., Eisele, L., Beier, F., Ziegler, P., Bauerschlag, D. O., Jöckel, K. H., Erbel, R., Mühleisen, T. W., Zenke, M., Brümmendorf, T. H. and Wagner, W. 2014. Aging of blood can be tracked by DNA methylation changes at just three CpG sites. *Genome Biol.* **15**: R24. [Medline] [CrossRef]
30. Yazawa, M., Okuda, M., Setoguchi, A., Iwabuchi, S., Nishimura, R., Sasaki, N., Masuda, K., Ohno, K. and Tsujimoto, H. 2001. Telomere length and telomerase activity in canine mammary gland tumors. *Am. J. Vet. Res.* **62**: 1539–1543. [Medline] [CrossRef]
31. Yi, S. H., Jia, Y. S., Mei, K., Yang, R. Z. and Huang, D. X. 2015. Age-related DNA methylation changes for forensic age-prediction. *Int. J. Legal Med.* **129**: 237–244. [Medline] [CrossRef]
32. Zubakov, D., Liu, F., van Zelm, M. C., Vermeulen, J., Oostra, B. A., van Duijn, C. M., Driessen, G. J., van Dongen, J. J., Kayser, M. and Langerak, A. W. 2010. Estimating human age from T-cell DNA rearrangements. *Curr. Biol.* **20**: R970–R971. [Medline] [CrossRef]