Gene Reports 23 (2021) 101022

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

Age estimation by DNA methylation levels in Iraqi subjects

Hiba S.G. Al-Ghanmy [a], Nihad A.M. Al-Rashedi [a,*], Asaad Y. Ayied [b]

[a] Department of Biology, College of Science, Al Muthanna University, Samawah, Iraq
[b] Department of Animal Production, College of Agriculture, Basrah University, Basrah, Iraq

ARTICLE INFO

Keywords:
Forensic genomics
Epigenetics
DNA methylation
Age prediction
Blood
Pyrosequencing

ABSTRACT

Epigenomic studies suggest that DNA methylation profiles can indicate chronological age, which is essential in forensic investigation. Here, we analyzed DNA methylation at CpG sites in five genes to investigate their potential to predict human age. Human blood DNA samples, obtained from Iraqi subjects, were subjected to bisulfite conversion and pyrosequencing of 27 CpG sites located in the *ELOVL2*, *TRIM59*, *KLF14*, *FHL2*, and *MIR29B2CHG* genes. An age prediction model based on multivariate regression analysis and leave-one-out cross-validation (LOOCV) was built using the three highest age-correlated loci present in *ELOVL2, TRIM59,* and *KLF14* genes, which provided a mean absolute deviation of 4.85 and 5.17, and root mean square error of 5.69 and 6.58 years. The results of ANOVA suggest that there is no significant difference between DNA methylation levels of males and females in the five genes. Finally, the new set of three methylated CpG markers is capable of producing an accurate age-predicted model of blood samples. Predicted age correlated well with chronological age in the 18–39 and 40–59 year age categories, but less accurately in the ≥60 year age category.

1. Introduction

Aging is a slow process associated with time-dependent changes in the physiological functions of a normal organism. Studies have reported the ability to measure age-related changes (Slieker et al., 2016). Multiple methods of age estimation have played an important role in forensic investigation and medical pathology. For example, skeletal examination of bones and teeth can be used to estimate age. However, the relationship between skeletal/dental age and actual age is not linear, but actually an indication of the developmental stage of the individual, which is poorly correlated with chronological age. Moreover, the examination required to analyze a full sample of tooth or bone requires a highly trained and experienced specialist. The applicability of these methods is limited owing to inter-observer errors of osteon levels and the absence of a global standard for this method (Wolff et al., 2012; Cunha et al., 2009). Most of these limitations also apply to investigation of tooth samples (Alkass et al., 2010). These limitations led to a search for more accurate methods. More recent studies have indicated that immunological parameters (De Toda et al., 2016) and lengths of telomeres (Karlsson et al., 2008) are useful markers in age determination, although they have high errors. Mitochondrial DNA is thought to be affected by oxidative damage caused by aging. Thus, deletion and heteroplasmy in mitochondrial DNA are molecular-genetic markers used in age determination, allowing differentiation between young and elderly individuals in forensic practice (Calloway et al., 2000). Although the aging process has long been associated with modifications in tissues and organs at a molecular level, it was only recently associated with epigenetic changes in DNA methylation.

Forensic DNA phenotyping aims to infer the appearance of an unknown sample donor from DNA when there is no suspect to match or when the DNA profile from the evidence does not match anyone in the forensic database. Forensic DNA phenotyping is expected to provide investigative leads that facilitate a search for an unknown suspect or a missing person by narrowing the search range (Lee et al., 2016).

Epigenetics, on the other hand, is the study of reversible, yet heritable, mechanisms, particularly DNA methylation, that occur without any alteration of the underlying DNA sequence. Although chromosomes in our genome carry the genetic information, the epigenome influences the functional use and stability of that information; that is, it connects the genotype with the phenotype (Johansson et al., 2013). Epigenetic forensic assays do not perform the same forensic functions as genetic assays; however, they may allow age determination. Studies involving identical twins have shown an association between epigenetic drift and aging, such that global methylation levels change during aging (Fraga and Esteller, 2007), although specific local CpG sites can become either hypo- or hyper-methylated over time (Florath et al., 2014).

* Corresponding author at: Department of Biology, College of Science, Al Muthanna University, Iraq.
E-mail address: nhidaee@mu.edu.iq (N.A.M. Al-Rashedi).

https://doi.org/10.1016/j.genrep.2021.101022
Received 4 October 2020; Received in revised form 4 December 2020; Accepted 29 December 2020
Available online 19 January 2021

Methylation profiles of CpG sites have been used for age estimation (Horvath, 2013). An age estimate resulting from a multivariable regression and leave-one-out cross-validation models of epigenetic DNA methylation profiles is referred to as epigenetic age. Several studies have reported age prediction models with an emphasis on novel methylation markers. However, we attempted to find new epigenetic markers that have a correlation with chronological age in the *ELOVL2*, *TRIM59*, *KLF14*, *FHL2*, and *MIR29B2CHG* genes, proposed by Zbieć- Piekarska et al. (Zbiec-Piekarska et al., 2015), in an Iraqi population.

2. Methods and materials

2.1. Blood samples

Peripheral blood samples ($n = 92$) were collected from 44 male and 48 female healthy Arab Iraqi between 18 and 93 years of age (mean age 54.3 years, median age 55.5 years). The study was approved by the ethics committee of the College of Science of Al-Muthanna University (Document number 4229 at 10/24/2017). All participants provided signed informed consent, and age information was obtained from the identity of civil status or the certificate of Iraqi nationality issued by the Directorate of Iraqi citizenship.

2.2. DNA extraction

Automated genomic DNA purification from blood samples was carried out using a DNA extraction machine Magtration System 12GC Plus and the Whole Blood MagDEA DNA 200 (GC) kit (PSS, Japan). The concentration and purity of the resulting DNA was measured using a Nanodrop-1000 UV-VIS spectrophotometer (Thermo Fisher Scientific Inc., USA).

2.3. Bisulfite treatment of genomic DNA

Bisulfite modification of 500 ng of extracted genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research Inc., USA), according to the manufacturer's protocol. Each assay included an internal control for completion of bisulfite modification of input target sequence.

2.4. Polymerase chain reaction (PCR)

PCR was performed using Qiagen HotStar Taq Polymerase. The reaction consisted of 1 μl of DNA template and 0.2 μM of each primer (one biotinylated), and 20 ng of bisulfite-treated DNA in a final reaction volume of 30 μl.

The final PCR product was purified using sepharose beads. PCR primers for the human *ELOVL2*, *TRIM59*, *KLF14*, *FHL2*, and *MIR29B2CHG* genes were designed by and purchased from EpigenDx (USA). The locations of primers and PCR product sizes, target DNA sequences, component reaction of PCR, and PCR conditions are shown in Supplemental Information SU1, Tables S1, S2, and S3.

2.5. Pyrosequencing

PCR products were bound to streptavidin sepharose High Performance columns (GE Healthcare, USA). The Sepharose beads with the biotinylated PCR products were then purified, washed, and denatured using a 0.2 M NaOH solution, then rewashed using the Pyrosequencing Vacuum Prep Tool (Qiagen) according to the manufacturer's protocol. PCR products were annealed with pyrosequencing primers and sequenced by using the PSQ96 HS System (QiagenF Inc., USA), according to the manufacturer's instructions. The methylation status of each locus was analyzed individually as a T/C single-nucleotide polymorphism (SNP) using Q-CpG software (Qiagen). Methylation scores were calculated as the percentage of methylated alleles at each CpG site divided by the total number of methylated and unmethylated alleles. Mean methylation rates were calculated using methylation percentages at CpG sites of each gene.

2.6. PCR bias testing

PCR bias testing was performed by mixing unmethylated control DNA with in vitro-methylated DNA at different ratios (0%, 5%, 10%, 25%, 50%, 75%, and 100%), as shown in Supplemental Information SU2, Figs. S1, S2, S3, S4, and S5. PCR bias testing was carried out for the human *ELOVL2*, *TRIM59*, *KLF14*, *FHL2*, and *MIR29B2CHG* genes. The percent methylation determined from the assay was highly correlated with expected methylation percentages, with an R^2 value of 0.90 to 0.99, thereby validating all five assays.

2.7. Statistical analysis

The simple linear regression was used to check the fitting degree of each CpG site that belongs to a certain gene. The Pearson correlation coefficients used to compare the effect sizes of age-associated sites. Finally, multivariate linear regression analysis was applied to generate age prediction models based on select CpG sites with the highest adjusted coefficient of determination ($R2\ ^{(}adj^{)}$) as well as *P* values. The Mean Absolute Deviation (MAD) and root mean square error (RMSE) were measured to assess the accuracy of models. In order to evaluate the predictive accuracy of the model, leave one out cross-validation (LOOCV) was performed. High R^2 and small MAD and RMSE values suggest the efficiency and fidelity of the chronological age prediction model. For statistical significance, analysis of variance (ANOVA) was conducted to assess the relationship between methylation values and gender. All statistical analyses were performed using MiniTab18 software.

3. Results

The methylation data were derived from amplification of CpG islands within the *ELOVL2*, *TRIM59*, *KLF14*, *FHL2*, and *MIR29B2CHG* genes using bisulfite conversion-pyrosequencing. The methylated CpG sites investigated were 27 CpG sites [Supplemental Information SU3]. The linear correlation between age and the DNA methylation level of the CpG sites was measured. Of these, 19 were hypermethylated for *ELOVL2*, *TRIM59*, *KLF14*, and *FHL2,* whereas 8 were hypomethylated for *MIR29B2CHG* [Supplemental Information SU2, Tables S1-S10 and Figs. S6-S10]. The *ELOVL2* gene showed nine CpG sites with the strongest correlations and significant hypermethylation linearity with chronological age (from $R = 0.833$ to 0.919). All CpG sites in *ELOVl2* gene clearly increased DNA methylation during age [Table 1]. The *TRIM59* gene had four hypermethylated CpG sites with significant linear relationships with age; the methylation levels increased with age but Pearson r values were lower than those observed for *ELOVL2*. As illustrated in Table 1, there were slight differences in the strength of the age association among these four CpG sites (CpG1, CpG2, CpG3 and CpG4 were $R = 0.749$, 0.769, 0.766, and 0.772, respectively).

Similar to *TRIM59*, the *KLF14* gene also the analyzed region contained four sites. All four CpG sites of *KLF14* showed hypermethylation, with CpG3 showing the strongest correlation with age ($R = 0.727$). In addition, although *KLF14* sites showed significant linear relationships with age in a simple regression model, the correlation coefficients were less than those for *ELOVL2* and *TRIM59*. For *FHL2*, two CpG sites were observed to show positive moderate linear correlations with age ($R = 0.579$ for CpG1 and $R = 0.461$ for CpG2). However, the correlations were weaker than those observed for *ELOVL2*, *TRIM59*, and *KLF14*.

The assay for *MIR29B2CHG* indicated two potential regions [Supplemental Information SU1, Table S1], the first, located in intron 4, at Chr1:207823822–207,823,845. This region contained four sites [Table 1]. The second region located downstream at Chr1: 207801998-

Table 1
Correlation analysis of CpG sites in the *ELOVL2, TRIM59, KLF14, FHL2, and MIR29B2CHG* genes as reported for the Arab Iraqi population in this study.

Gene	Site	Assay location	From ATG	From TSS	GRCh38	R^2(adj)	Pearson correlation
ELOVL2	CpG1	Intron 1	869	869	Chr6:11044628	0.781	0.885**
	CpG2	Intron 1	872	872	Chr6:11044631	0.804	0.898**
	CpG3	Intron 1	875	875	Chr6:11044634	0.837	0.916**
	CpG4	Intron 1	881	881	Chr6:11044640	0.821	0.907**
	CpG5	Intron 1	883	883	Chr6:11044642	0.772	0.880**
	CpG6	Intron 1	885	885	Chr6:11044644	0.842	0.919**
	CpG7	Intron 1	888	888	Chr6:11044647	0.816	0.905**
	CpG8	Intron 1	896	896	Chr6:11044655	0.691	0.833**
	CpG9	Intron 1	902	902	Chr6:11044661	0.747	0.866**
TRIM59	CpG1	Promoter	−11,166	−520	Chr3:160450349	0.557	0.749**
	CpG2	Promoter	−11,172	−526	Chr3:160450355	0.588	0.769**
	CpG3	Promoter	−11,179	−533	Chr3:160450362	0.587	0.766**
	CpG4	Promoter	−11,182	−536	Chr3:160450365	0.593	0.772**
KLF14	CpG1	Promoter	−340	−312	Chr7:130734373	0.518	0.725**
	CpG2	Promoter	−343	−315	Chr7:130734376	0.461	0.683**
	CpG3	Promoter	−366	−338	Chr7:130734399	0.524	0.727**
	CpG4	Promoter	380-	−352	Chr7:130734413 G > A rs7808283	0.253	0.511**
FHL2	CpG1	Intron 1	−12,742	39,246	Chr2:105399258	0.329	0.579**
	CpG2	Intron 1	−12,733	39,255	Chr2:105399249	0.205	0.461**
MIR29B2CHG	CpG1	Intron 4	N/A	55,252	Chr1:207823845	0.120	−0.360**
	CpG2	Intron 4	N/A	55,264	Chr1:207823833	0.036	−0.215*
	CpG3	Intron 4	N/A	55,268	Chr1:207823829	0.280	−0.536**
	CpG4	Intron 4	N/A	55,275	Chr1:207823822	0.033	−0.209*
	CpG5	Downstream	N/A	77,078	Chr1:207802019	0.000	−0.102
	CpG6	Downstream	N/A	77,081	Chr1:207802016	0.035	−0.212*
	CpG7	Downstream	N/A	77,090	Chr1:207802007	0.000	0.005
	CpG8	Downstream	N/A	77,099	Chr1:207801998	0.031	−0.203

* Correlation significant at the 0.05 level (2-tailed).
** Correlation significant at the 0.01 level (2-tailed).

207802019, also contained four sites. Most CpG sites in *MIR29B2CHG* appeared to have a negative linear relationship with age, i.e., all sites were less methylated with chronological age. Furthermore, the fitting process did not provide satisfactory coefficients of determination [Table 1].

The best three CpG sites from CpG6 in *ELOVL2*, CpG4 in *TRIM59*, and CpG3 in *KLF14* genes were tested in a multivariate regression model to assess age estimations. The mean absolute deviation (MAD) and root mean square error (RMSE) between the predicted and the observed ages was 4.85 and 5.69 years, respectively (all P- values <0.05 and R^2 (adj) = 0.842) [Supplemental Information SU2, Table S11A] [Fig. 1].

The leave-one-out cross-validation (LOOCV) was performed by using three predictors CpG6 in *ELOVL2*, CpG4 in *TRIM59*, and CpG3 in *KLF14* which was fit on all but one subject and the age of the left-out subject is then predicted with this model. A complete LOOCV, meaning that the procedure was repeated for 92 times so that each subject served as a validation set, resulted in MAD of 5.17 and RMSE of 6.58 years [Supplemental Information SU2, Table S11B] [Fig. 2].

Next, individuals were grouped into three age categories: 18–39 (n = 26), 40–59 (n = 24), and ≥ 60 (n = 42) years. To evaluate age

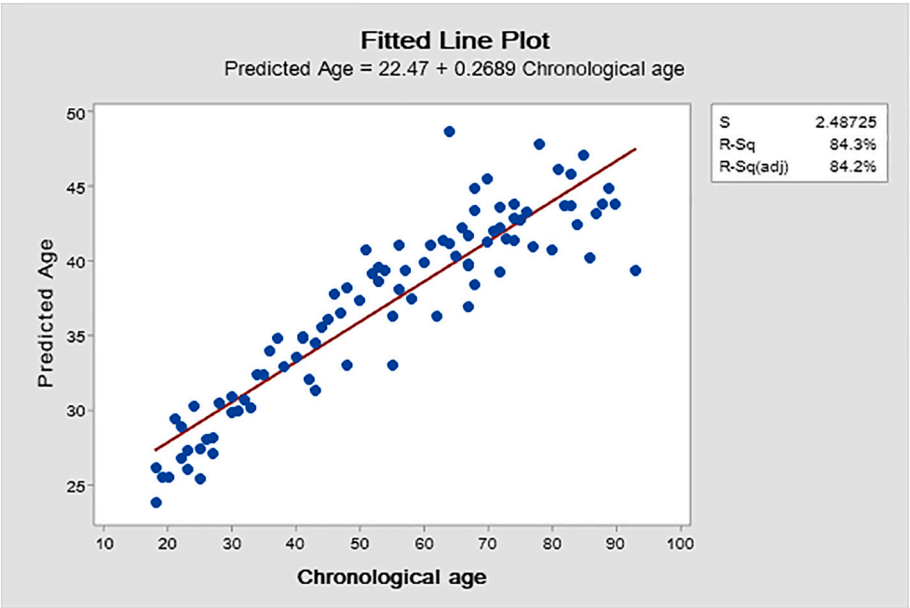

Fig. 1. Predicted versus chronological age for CpG6 in *ELOVL2*, CpG4 in *TRIM59*, and CpG3 in *KLF14* in 92 blood samples with MAD = 4.85, RMSE = 5.69.

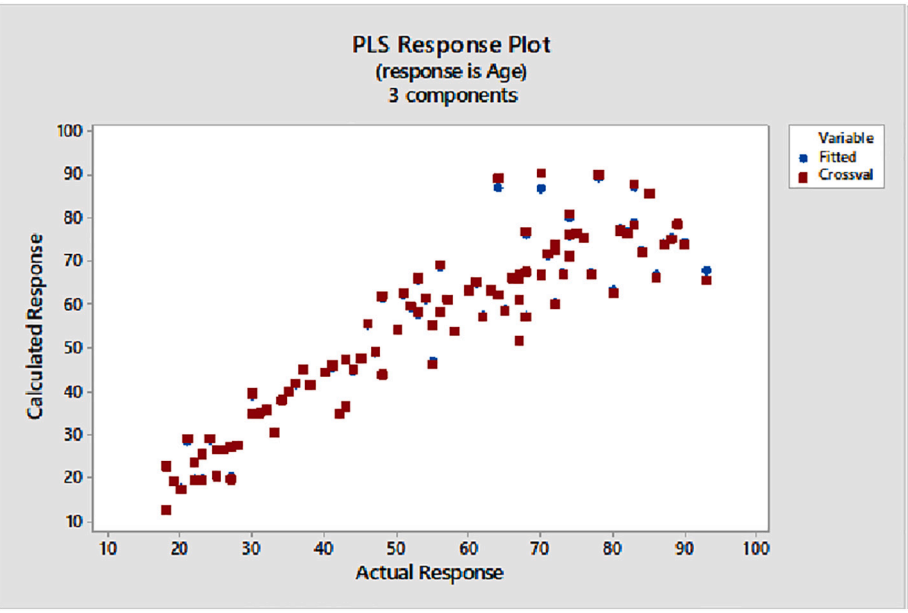

Fig. 2. Predicted versus chronological age of blood samples using a leave-one-out model was based on three predictors: CpG6 in *ELOVL2*, CpG4 in *TRIM59*, and CpG3 in *KLF14* versus observed age. The predicted values were highly correlated with the observed ages, with MAD = 5.17, RMSE = 6.58.

prediction, DNA methylation levels of blood samples were determined by using multivariate regression model and leave-one-out cross-validation of three CpG sites in the *ELOVL2, TRIM59,* and *KLF14* genes on the basis of these three age categories. We observed different accuracies when comparing predicted age with chronological age categories using the MAD and RMSE scales of the various age groups. The prediction results were significantly better for samples from the category of 19–39 years (MAD = 1.8, RMSE = 2.4 and 2.6) than for the category of 40–59 years (MAD = 3.0 and 3.3, RMSE = 3.8 and 4.1), and the category of ≥60 years was less significant for estimating predicted age (MAD = 6.0 and 4.8, RMSE = 7.6 and 6.4) [Supplemental Information SU2, Table S12A & B].

There were no significant differences in methylation levels between men and women for any CpG marker. Thus, we can conclude that in this study, sex does not play a significant role in age-associated methylation [Supplemental Information SU2, Tables S13-S17].

4. Discussion

In this study, the methylation levels assessed for 27 CpG sites located in five genes were shown to be associated with chronological age and the data were used to make a model of age prediction from DNA methylation data. We optimized the correlation analysis with different 27 CpG sites from five genes to find a set of the highest age-correlated sites present in three CpG sites from ELOVL2, TRIM59, and KFL14. The rationale reason to the most correlated CpG site from each gene was selected for the age prediction model construction because methylation status at adjacent sites tends to be correlated and to avoid biased results (Shoemaker et al., 2010).

The resulting multivariate regression model was able to predict donor age with a mean error of 4.85 years. These results are consistent with a recent studies executed to find the relationship between multiple methylation markers in the human genome and chronological age (Parson, 2018). Therefore, these loci provide an appropriate selection for age prediction modeling.

The biomarkers used in a prediction model should show large changes in methylation over the course of human life and show low errors for age prediction (Vidaki et al., 2017). In this study, *ELOVL2* was highly correlated with age and showed hypermethylation of nine CpG sites with aging. In addition, all CpG sites gave good results in a multiple regression model. These results are consistent with those of a Singaporean population (Thong et al., 2017), but only seven CpG sites have been identified in a Polish (Zbiec-Piekarska et al., 2015) and Korean (Cho et al., 2017) populations. Previously, Bacalini et al. reported that DNA methylation of *ELOVL2* CpG islands from whole-blood DNA samples was highly correlated with age and demonstrated that most tissues show *ELOVL2* hypermethylation with age (Bacalini et al., 2016).

The methylation status of CpG islands in the promoter of *TRIM59* was also effectively correlated with age, consistent with the results reported by Zbieć-Piekarska et al. (Zbiec-Piekarska et al., 2015).

Methylation at all CpG sites of the *KLF14* gene had significant linear relationships with age, which was consistent with the results of Cho et al., who found that methylation at the four CpG positions of *KLF14* was median correlated with chronological age in Koreans and they could explain age related variance by 20.7 to 36.8% (Cho et al., 2017).

We evaluated the association between age and methylation for two CpG sites located in *FHL2* within the CpG islands of intron 1 and found that correlation between the methylation status at these loci and age was weak. Therefore, methylated CpG sites (Chr2:105399258 and Chr2:105399249) at the *FHL2* gene were not considered to be the best age predictor. In contrast, the DNA methylation of *FHL2* CpG sites in blood and saliva samples showed a very strong positive correlation with age ($R > 0.7$) (Jung et al., 2019). The results of Steegenga et al. reported two genomic regions within the CpG islands of the promoters of *FHL2* and *ELOVL2* whose methylation status was highly correlated with age. Both *FHL2* and *ELOVL2* showed continuous increase in methylation levels with age, with methylation values ranging from 12% to 53% for *FHL2* and from 7% to 91% for *ELOVL2 (*Steegenga et al., *2014)*.

By contrast, CpG sites belonging to the *MIR29B2CHG* gene had a negative linear relationship with age. However, the fitting process for *MIR29B2CHG* did not provide satisfactory coefficients of determination. In addition, our results for *MIR29B2CHG* were in disagreement with previous reports, likely because the identified regions in our study were different from those in the other studies (Cho et al., 2017; Freire-Aradas et al., 2018).

These results are in slight disagreement with previous reports, with the exception of the *ELOVL2* gene. This gene has the same target methylated markers described in previous studies (Zbiec-Piekarska et al., 2015; Thong et al., 2017; Cho et al., 2017; Bacalini et al., 2016), and, unlike the other four genes *TRIM59*, *KLF14*, *FHL2*, and

MIR29B2CHG, presented new targeted methylated regions. We excluded the methylated CpG sites of the *FHL2* and *MIR29B2CHG* genes from the age predictive model because the statistical analysis methylated CpG sites of the FHL2 and MIR29B2CHG genes using R^2 showed weakly correlated with chronological age.The age predictive model provided MAD = 4.85 and 5.17 years, and age category models MAD = 1.85, 3.01 and 3.31, and 6.07 and 4.82 years for 18–39, 40–59, and ≥ 60 years, respectively. Remarkably, the CpG sites identified in this study were different from those reported in other populations. These populations were distant from the communities of the Middle East, and the age range of the subjects in this study was greater than that of subjects in previous studies.

5. Conclusions

In this study, we have shown that methylation of three CpG sites in the *ELOVL2, TRIM59*, and *KLF14* genes had an effective linear relationship with age; this enabled the prediction of chronological in Arab Iraqi adults with high accuracy. Pyrosequencing to measure DNA methylation provides a useful tool for forensic age estimation from blood samples. Further studies are necessary to assess epigenetic variation in other ethnic populations to control for genetic and environmental influences on methylation levels before the widespread adoption of DNA methylation for forensic age determination.

Abbreviations

ANOVA	Analysis of Variance
CpG	Cytosine-phosphate-Guanine
DNA	Deoxyribonucleic Acid
ELOVL2	Fatty Acid Elongase 2
FHL2	Four and a half LIM domains protein 2
KLF14	Krüppel-like factor 14
LOOCV	leave-one-out cross-validation
MAD	Mean Absolute Deviation
RMSE	Root Mean Square Error
PCR	Polymerase chain reaction
TRIM59	Tripartite Motif-containing Protein 59

Funding

None.

Research involving human participants and/or animals

The study was approved by the ethics committee of the College of Science of Al-Muthanna University (Document number 4229 at 10/24/2017).

Informed consent

All participants provided signed informed consent.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

CRediT authorship contribution statement

Nihad Al-Rashedi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing - original draft, Review & Editing. **Hiba S.G. Al-Ghanmy:** Data curation, Investigation, Methodology, Resources, Writing - original draft. **Asaad Y. Ayied**: Formal analysis, Investigation, Software, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.genrep.2021.101022.

References

Alkass, K., Buchholz, B.A., Ohtani, S., Yamamoto, T., Druid, H., Spalding, K.L., 2010. Age estimation in forensic sciences: application of combined aspartic acid racemization and radiocarbon analysis. Mol. Cell. Proteomics 9 (5), 1022–1030. https://doi.org/10.1074/mcp.M900525-MCP200.

Bacalini, M.G., Deelen, J., Pirazzini, C., De Cecco, M., Giuliani, C., Lanzarini, C., et al., 2016. Systemic age-associated DNA hypermethylation of *ELOVL2* gene: in vivo and in vitro evidences of a cell replication process. J. Gerontol. A Biol. Sci. Med. Sci. 72 (8), 1015–1023. https://doi.org/10.1093/gerona/glw185.

Calloway CD, Reynolds RL, Herrin GL Jr, Anderson WW. The frequency of heteroplasmy in the HVII region of mtDNA differs across tissue types and increases with age. Am J Hum Genet.2000; 66:1384-97. 0002-9297/2000/6604-0020$02.00.

Cho S, Jung SE, Hong SR, Lee EH, Lee JH. Lee SD et al. Independent validation of DNA-based approaches for age prediction in blood. Forensic Sci Int.2017; Genet 29:250–6. doi:https://doi.org/10.1016/j.fsigen.2017.04.020 -.

Cunha E, Baccino E, Martrille L, Ramsthaler F, Prieto J, Schuliar Y et al. The problem of aging human remains and living individuals: a review. Forensic Sci Int. 2009; 193 (1–3):1–13.https://doi.org/10.1016/j.forsciint.2009.09.008.

De Toda IM, Maté I, Vida C, Cruces J, De la Fuente M. Immune function parameters as markers of biological age and predictors of longevity. Aging (Albany NY).2016; 8 (11):3110–9. 10.18632/aging.101116.

Florath, I., Butterbach, K., Muller, H., Bewerunge-Hudler, M., 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. https://doi.org/10.1093/hmg/ddt531.

Fraga, M.F., Esteller, M., 2007. Epigenetics and aging: the targets and the marks. Trends Genet. 23, 413–418. https://doi.org/10.1016/j. tig.2007.05.008.

Freire-Aradas A, Phillips C, Girón-Santamaría L, et al. Tracking age-correlated DNA methylation markers in the young. Forensic Sci Int Genet.2018;. 36:50–59. doi:https://doi.org/10.1016/j.fsigen.2018.06.011.

Horvath, S., 2013. DNA methylation age of human tissues and cell types. Genome Biol. 14, R115. https://doi.org/10.1186/gb-2013-14-10-r115.

Johansson, A., Enroth, S., Gyllensten, U., 2013. Continuous aging of the human DNA methylome throughout the human lifespan. PLoS One 8, e67378. https://doi.org/10.1371/journal.pone.0067378.

Jung, S.E., Lim, S.M., Hong, S.R., Lee, E.H., Shin, K.J., Lee, H.Y., 2019. DNA methylation of the ELOVL2, FHL2, KLF14, C1orf132/MIR29B2C, and TRIM59 genes for age prediction from blood, saliva, and buccal swab samples. Forensic Sci Int Genet. 38, 1–8. https://doi.org/10.1016/j.fsigen.2018.09.010.

Karlsson, A.O., Svensson, A., Marklund, A., Holmlund, 2008. Estimating human age in forensic samples by analysis of telomere repeats. Forensic Sci Int Genet Suppl Ser. 1, 569–571. https://doi.org/10.1016/j.fsigss.2007.10.153.

Lee, H.Y., Lee, S.D., Shin, K.J., 2016. Forensic DNA methylation profiling from evidence material for investigative leads. BMB Rep. 49, 359–369. https://doi.org/10.5483/BMBRep.2016.49.7.070.

Parson, W., 2018. Age estimation with DNA: from forensic DNA fingerprinting to forensic (Epi) genomics: a mini-review. Gerontology. 64, 326–332. https://doi.org/10.1159/000486239.

Shoemaker, R., Deng, J., Wang, W., Zhang, K., 2010. Allele-specific methylation is prevalent and is contributed by CpG-SNPs in the human genome. Genome Res. 20 (7), 883–889. https://doi.org/10.1101/gr.104695.109.

Slieker, R.C., Van Iterson, M., Luijk, R., Beekman, M., Zhernakova, D.V., Moed, M.H., et al., 2016. Age-related accrual of methylomic variability is linked to fundamental ageing mechanisms. Genome Biol. 17, 191. https://doi.org/10.1186/s13059-016-1053-6.

Steegenga W, Boekschoten M.V, Lute, C., Hooiveld GJ, de Groot PJ, Morris TJ et al. Genome-wide age related changes in DNA methylation and gene expression in human PBMCs. Age (Dordr).2014;36(3):9648. https://doi.org/10.1007/s11357-014-9648-x.

Thong, Z., Chan, X.L.S., Tan, J.Y.Y., Loo, E.S., Syn, C.K.C., 2017. Evaluation of DNA methylation-based age prediction on blood. Forensic Sci Int Genet Suppl Series. 6, e249–e251. https://doi.org/10.1016/j.fsigss.2017.09.095.

Vidaki, A., Ballard, D., Aliferi, A., Miller, T.H., Barron, L.P., Syndercombe, Court D., 2017. DNA methylation-based forensic age prediction using artificial neural network

and next generation sequencing. Forensic Sci Int Genet. 28, 225–236. https://doi.org/10.1016/j.fsigen.2017.02.009.

Wolff K, Vas Z, Sotonyi P, Magyar LG. Skeletal age estimation in Hungarian population of known age and sex. Forensic Sci Int.2012; 223(1–3):374. e1–8.https://doi.org/10.1016/j.forsciint.2012.08.033.

Zbiec-Piekarska, R., Spolnicka, M., Kupiec, T., Parys-Proszek, A., Makowska, Z., Paleczka, A., et al., 2015. Development of a forensically useful age prediction method based on DNA methylation analysis. Forensic Sci Int Genet. 17, 173–179. https://doi.org/10.1016/j.fsigen.2015.05.001.