DNA Methylation as a Coming Clue Age Prediction

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Abstract

Background: *DNA methylation (DNAm) is a biochemical modification which occurs over the lifespan of an individual and it is a significant constituent in the aging process. The degree of methylation was significantly related to age. The locus ELOVL2 is the most thoroughly evaluated marker of age so far. It has been shown that this locus can be reliably analyzed in old and fresh human bloodstains, which are a main source of DNA in forensic laboratories.* ***Aim of work:*** *The current study aimed to assess the use of DNA methylation on the ELOVL2 gene from blood samples as biomarkers for chronological age estimation using pyrosequencing in Egypt.* ***Material and methods****: 80 whole blood samples from individuals aged 18-96 years divided into 4 groups were analysed using a DNA methylation quantification assay based on bisulphite conversion and DNA pyrosequencing of 7 CpG sites in the ELOVL2. Our results display significantly strong correlation between DNAm and chronological age; the model supporting DNAm as a strong age predictor. The age prediction accuracy was most accurate in age group III* ***(****40-49 y****)*** *and was least accurate in age group IV of the elderly individuals (50-69 y) on choosing1, 2.5-, and 5-years as difference threshold.*

Key words: *DNA methylation, aging, whole blood, ELVOL2.*

1. INTRODUCTION

Aging is a natural and gradual process in human life. The chronological age, the time elapsed since birth, is different from the biological age which refers to how old a person seems influenced by genetic and environmental factors such as diseases, lifestyle and social factors independent of the passage of time alone (Freire-Aradas et al., 2017; Jung et al., 2017). Age discrimination of anonym human bodies is an important issue in the field of forensic medicine. It can provide valuable information to the medicolegal interrogator in crime inquiry as well as utility in mass catastrophe situations where age may be difficult to estimate (Elmadawy et al., 2021). Estimation of chronological age from biological materials such as bloodstain is a pivotal important point in forensic investigations (Meissner and Ritz-Timme, 2010), procedure based on forensic genetic analysis expected to provide some advantageous information than conventional methods of age estimation as there is no adequate morphologic or biochemical information (Ou et al., 2012). Since then, new DNA tests have been investigated to deduce individual age from a biological trace (Freire-Aradas et al., 2017).

The use of DNA methylation (DNAm) to obtain additional information in forensic investigations showed to be a promising and increasing field of interest (Naue et al., 2017). For a

long time, DNAm was a “black spot” for forensic scientists, but now it can give additional forensic relevant information in parallel to the DNA profile (Vidaki et al., 2017). DNAm involves the addition of a methyl group to the 5’ cytosine of CG dinucleotide, referred to as CpGs and related to gene regulation and cell differentiation by affecting transcription factor binding sites, insulator components, and chromosome morphology (Ziller et al., 2013). These functions explain why DNAm at specific sites in the genome shows a cell-type specific pattern and how it can be used for tissue/body-fluid discrimination (Lee et al., 2012). Recent progress in epigenomics allowed identification of multiple DNA methylation loci that can be useful for age prediction. Some of these markers have been used to develop age prediction models that may have a practical value in forensics. It has been shown that DNA methylation markers outperform the other types of potential age predictors including age-shortening telomeres, age-dependent changes in T cells’ DNA, and age-altering mRNA level (Spólnicka et al., 2017). In the forensics field, the promoter of ELOVL2 gene is considered the most promising locus for age prediction (Park et al., 2016).

Forensic genetics have been recently burgeoned in Egypt. Until recently, the data about age prediction in Egyptians by using molecular genetics methods still finite. Therefore, Egyptian forensic DNA data extension is essentially recommended. So, the current study aimed to use of DNA methylation from blood samples for chronological age prediction. The novelty in this study lies in the application of this type of epigenetic studies on an Egyptian population which was not explored previously, and we were interested in investigating whether established methods and results apply to the Egyptian population.

2. MATERIALS AND METHODS

2.1. Sample collection, DNA extraction and Quantification:

-A total of 80 healthy unrelated volunteers of both sexes were included in this study. The volunteer's age ranged from 18–69-year-old which categorized into 4 groups as following:

Group (1): 18 – 29 years.

Group (2): 30 – 39 years.

Group (3): 40 – 49 years.

Group (4): 50 – 69 years.

-Inclusion criteria: All are healthy unrelated Egyptian donors aged from 18 – 69 years.

-Exclusion criteria: Individuals with age-associated disease (such as alzheimer’s disease, parkinson’s disease, cardiovascular disease, immune disease and impaired cognitive functions), human genetic

syndromes (such as Down syndrome and Werner's syndrome), alcohol drinking, smoking. obesity, metabolic syndrome and cancer.

-Whole blood samples were collected from all subjects into EDTA tubes to be stored at -80^{o}C for further molecular assay. Genomic DNA was extracted from the collected blood samples using the DNA isolation kit (G-spin^{TM}, Korea) according to the manufacturer instructions. DNA was then quantified and evaluated using nano drop spectrophotometer and gel electrophoresis to check the quality of the amplicon pool of each individual.

Written informed consents were obtained from them for their legally authorized representative. This study was approved by the Benha University Research Ethics Committee, approval number 00084

2.2.Bisulfite conversion and quantification

The extracted DNA was subjected to bisulfite conversion using Thermo Scientific™ EpiJET™ Bisulfite Conversion Kit.

2.3.PCR amplification and methylation analysis

PCR Amplification of DNA was done using COSMO PCR RED M. Mix and primer set. The Primer sequence was Biotin-AGGGGAGTAGGGTAAGTGAGG (sequence forward), AACAAAACCATTTCCCCCTAATAT (sequence reverse) and ACAACCAATAAATATTCCTAAAACT (sequencing).

PCR product purification and Agarose Gell Electrophoresis were done to check the quality of the product. DNA Pyrosequencing: The final DNA pool will be sequenced:

We act on ELVOL2 (ELVOL fatty acid elongase 2 chromosome location):

- CpG1: Chr6:11,044,661.
- CpG2: Chr6:11,044,655.
- CpG3: Chr6:11,044,647.
- CpG4: Chr6:11,044,644.
- CpG5: Chr6:11,044,642.
- CpG6: Chr6:11,044,640.
- CpG7: Chr6:11,044,634.

Lastly this formula was used to obtain intact result:

Zbiec-Piekarsa 1-42.8393176902677 + 0.63266203860581 × ELVOL2 (CPG5) + 0.877474742612866 × ELVOL2 (CPG7) (Zbieć-Piekarska et al., 2015).

2.4. Statistical Analysis

After data collection, data was revised, coded, and fed to statistical software IBM SPSS version 21. All values at $P \leq 0.05$ were considered to be significant. Data are presented as Minimum, Maximum, mean ± SE. Comparison of the correlation coefficient of the two population means of independent samples was done using the Student's T-test. Correlations among variables were studied by using the Pearson`s coefficient. The mean absolute deviation (MAD) and the standard error of estimate (SEE) were used to check the accuracy of predictions made with the regression line.

3. RESULTS

Blood-based age prediction model using pyrosequencing for DNAm analysis of 7 CpG sites from ELOVL2 gene was selected for evaluation of blood samples from 80 donors aged between 18 and 69 years divided into four age groups using Zbiec-Piekarska 1 model. There was no statistically significant differences between chronical age and estimated age in all age groups (Table 1). The mean of absolute value of predicted age minus chronological age in the four age prediction models ranged from 1.12 to 1.63 years, while the median value ranged from 0.11 to 1.96 years (Fig. 1).

Considering intergroup comparisons assessment was performed using the values of the predicted age minus chronological age of each group. There were no statistically significant differences between groups regarding mean values of age differences (Table 2).

Correlation analysis indicated a strong positive statistically highly significant correlation present overall ($0.790 \leq r \leq 0.892$, mean absolute $r = 0.826$) between predicted and chronological age for the four groups, which explained 62.4% to 79.6% of the age variation. The Pearson's correlation coefficients of the CpGs included in age prediction model were very similar between different age groups (Fig. 2).

The performance and accuracy of the age prediction model were evaluated by calculating the mean absolute deviation (MAD), the standard error of estimate (SEE) and the percentage of correct predictions (PCP), considering a difference of 1, 2.5 and 5 years between the predicted and chronological ages for all individuals, as well as for the four groups based on their chronological age. The model presented with the least performance observed in group IV (MAD of 3.932 and SEE of 4.816). When a threshold of 1 year difference was chosen, the age prediction accuracy was better in age group III (70% of correct predictions) than in age group I (65% of correct prediction) and in age groups II, IV (60% for both), on choosing 2.5 years difference as threshold, the age prediction accuracy was better in age group III (80% of correct predictions) than in age group I (75% of correct predictions) and in age groups II and in IV (70% of correct predictions for both), on choosing 5 years difference as threshold, the age prediction accuracy was better in age groups I and III (85% of correct predictions for both) than in age group IV (80% of correct predictions) and in age group II (75% of correct predictions) (Table 3).

The average and SE of DNA methylation of ELOVL2 gene at CpGs sites in blood samples at different age levels increase as age increased at different CpGs sites (Table 4). Correlation analysis indicated an excellent positive statistically highly significant correlation present between DNAm status at CpG(s) sites of ELOVL2 gene and blood estimated age (Table 5).

Table (1): Descriptive statistics of chronological age and estimated age in the four age-groups prediction model.

Groups	**All (18-69 y) (n=80)**	**Group I (18-29 y) (n=20)**	**Group II (30-39 y) (n=20)**	**Group III (40-49 y) (n=20)**	**Group IV (50-69 y) (n=20)**
Chronological age					
Mean ± SE	40.35±1.52	23.70±0.85	34.50±0.66	44.50±0.66	58.70±1.40
Median	39.50	24.00	34.50	44.50	58.00
Min-Max	18.0-69.0	18.0-29.0	30.0-39.0	40.0-49.0	50.0-69.0
Estimated age					
Mean ± SE	40.09±1.22	24.93±0.78	35.60±0.84	46.10±0.84	57.62±1.52
Median	39.04	26.05	33.21	45.61	57.09
Min-Max	12.52-69.57	12.52-34.75	29.53-42.68	37.71-49.90	48.18-69.57
Test of Significance (P-value)	0.949	0.223	0.230	0.172	1.974
	0.345	0.829	0.821	0.865	0.063

SE: Standard error

Table (2): Intergroup comparisons assessment using the values of the predicted age minus chronological age of each group.

Groups	**Group I vs II**	**Group I vs III**	**Group I vs IV**	**Group II vs III**	**Group II vs IV**	**Group III vs IV**
T test	0.918	0.856	0.283	0.118	0.874	1.258
P-value	0.308	0.624	0.778	0.907	0.388	0.184

Table (3): Evaluation of the accuracy of the age prediction models.

Individuals	All (n=80)	Group I (n=20)	Group II (n=20)	Group III (n=20)	Group IV (n=20)
MAD	**3.343**	**2.136**	**1.917**	**3.188**	**3.932**
SEE	**4.422**	**3.756**	**3.680**	**4.003**	**4.816**
PCP					
≤ 1 years	**63.8%**	**65%**	**60%**	**70%**	**60%**
≤ 2.5 years	**76.3%**	**75%**	**70%**	**80%**	**70%**
≤ 5 years	**82.5%**	**85%**	**75%**	**85%**	**80%**

MAD: Mean absolute deviation, SEE: Standard error of estimate, PCP: Percentage of correct predictions

Table 4: Average and SE of DNA methylation of ELOVL2 gene at CpG sites in blood samples at different age levels

ELOVL2 site	Group I (18-29 years) (n=20)	Group II (30-39 years) (n=20)	Group III (40-49 years) (n=20)	Group IV (50-69 years) (n=20)
CpG_1	67.70±0.44	74.74±0.50	82.02±0.53	89.72±0.53
CpG_2	47.56±0.50	54.50±0.43	62.0±0.58	69.53±0.51
CpG_3	46.80±0.46	54.18±0.45	60.68±0.51	69.28±0.57
CpG_4	58.06±0.50	65.15±0.47	71.87±0.47	79.99±0.49
CpG_5	33.54±0.58	40.10±0.48	46.44±0.69	55.05±0.97
CpG_6	22.26±0.47	29.22±0.47	35.53±0.48	44.30±0.84
CpG_7	51.49±1.06	59.34±0.65	66.16±0.62	74.79±1.17

*Values are expressed as Mean ± SE

Table 5: Correlation analysis between the DNA methylation status at different CpG(s) sites of ELOVL2 gene and estimated age.

CPG(s) sites	Estimated age				
	All (n=80)	**Group I (n=20)**	**Group II (n=20)**	**Group III (n=20)**	**Group IV (n=20)**
CpG_1	0.986 0.000*	0.902 0.000*	0.865 0.000*	0.788 0.000*	0.923 0.000*
CpG_2	0.984 0.000*	0.902 0.000*	0.862 0.000*	0.752 0.000*	0.929 0.000*
CpG_3	0.986 0.000*	0.902 0.000*	0.865 0.000*	0.788 0.000*	0.924 0.000*
CpG_4	0.986 0.000*	0.902 0.000*	0.865 0.000*	0.788 0.000*	0.923 0.000*
CpG_5	0.979 0.000*	0.922 0.000*	0.816 0.000*	0.832 0.000*	0.894 0.000*
CpG_6	0.983 0.000*	0.884 0.000*	0.826 0.000*	0.688 0.000*	0.925 0.000*
CpG_7	0.991 0.000*	0.983 0.000*	0.931 0.000*	0.934 0.000*	0.944 0.000*

*Significantly different at ($P<0.05$).

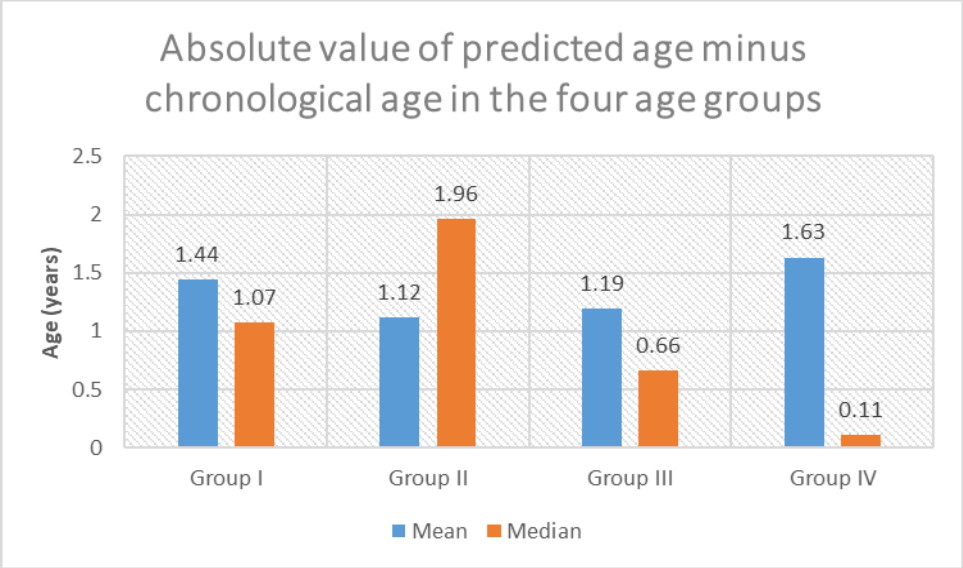

Figure (1): Absolute value of predicted age minus chronological age in the four-age group prediction model.

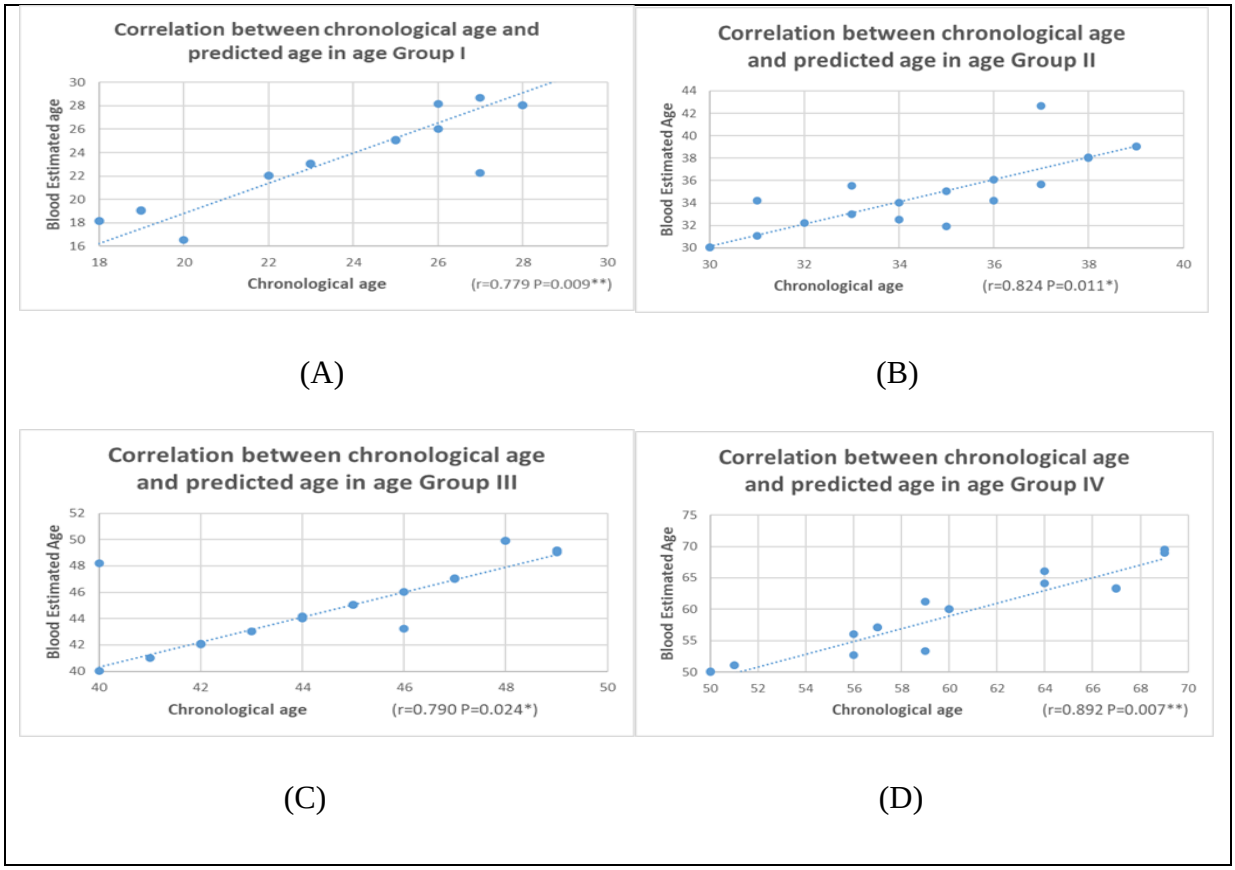

Figure (2): Correlation between chronological age and predicted age (A; age group I, B; age group II, C; age group III, D; age group IV).

4. DISCUSSION

For a long time, forensic science has been seeking a relevant type of marker facilitating age prediction from biological traces related to crime scene. Recently, DNAm is considered the most promising information source about human age in forensic science as the regulatory regions of several genes become progressively methylated with increasing age, suggesting a functional link between age, DNA methylation, and gene expression (Sukawutthiya et al., 2021).

The present study revealed a high correlation between predicted age and chronological age supporting DNAm as a strong age predictor. Remarkably, across a population, this DNAm age correlates strongly with chronological age (Field et al., 2018). Because of the high abundance of DNAm loci in the human genome which are linearly correlated with age and can match each other, providing a very accurate final estimate of chronological age (Zbieć-Piekarska et al., 2015). These results are matched with the results of previous studies (Huang et al., 2015; Jung et al., 2019; Mcewen et al., 2017; Xu et al., 2015). Also these findings are in accordance with those reported by, Daunay et al. (2019) who found strong correlation between DNAm of all CpGs and the chronological age of all individuals. In contrast, Jung et al. (2017) who found that methylation measures at several to hundreds of CpG sites are expected to measure biological age that is not always parallel with chronological age but instead may inform life expectancy predictions so DNA methylation age might therefore be useful in the research to identify age accelerator or decelerator for extended human life span.

In our data, the MAD between predicted and chronological age was largest in the categories of older people. This was in agreement with Bekaert et al. (2015) who found that, the MAD between predicted and chronological age was the largest for people ≥ 60 years old. Additionally, Zbieć-Piekarska et al. (2015) reported that age prediction accuracy is lowest in the age category comprising individuals aged 60–75 years. Also, this result is in concordance with previous reports confirming that DNAm patterns predict age with more accuracy in younger than in older individuals as older individuals showed an increased deviation, and their age is rather under predicted (Correia Dias et al., 2020; Naue et al., 2017). DNA methylation of ELOVL2 and age was not a straight line but rather increased exponentially during childhood, while flattening out during later life (Bekaert et al., 2015). This result may be explained by environmental factors contributing more significantly to the DNA methylation status in older individuals whose medical history or life style differences increase age estimation error (Zbieć-Piekarska et al., 2015). In contrast, Huang et al. (2015)

reported that, there is no significant difference between the adolescent group and the elderly group as they worked on a small sample size consisted of 10 blood samples from younger donors (aged from 10 to 25 years) and 10 blood samples from senior donors (aged from 55 to 65 years) to screen the candidate markers.

In the present study, the age prediction accuracy was more accurate in age group III **(**40-49 y**)** and less accurate in age group IV (50-69 y) on choosing 1, 2.5-, and 5-years difference as threshold. This result in accordance with Freire-Aradas et al. (2016) who found that Category III (40–59) was successfully predicted (76.47% of the population), when the predicted age matched the actual age 5 years. Also, in the same vein of Al-Ghanmy et al. (2021) who reported that predicted age correlated well with chronological age in the 40–59 year age categories, but less accurately in the ≥60 year age category. Also, in agreement with Bekaert et al. (2015) and Zbieć-Piekarska et al. (2015), who found that 55.2% success rate for samples of 60–75 years and 54.9% for study samples of 60–91 years, respectively. DNAm changes do not occur at a constant rate during a lifetime but accumulate rapidly up to adulthood (Freire-Aradas et al., 2016). Certain ageing-associated DNAm changes appear to be programmed, whereas others are caused by environmental and stochastic effects. Increased methylome age has been shown to be associated with decreased mental or physical fitness in elderly individuals and higher mortality in individuals aged 69-79 years (Marttila, 2016).

The present study illustrated that ELOVL2 seems to be a very promising candidate marker for age estimates. Freire-Aradas et al. (2016) confirmed that ELOVL2 has been widely reported as a principal age predictor and therefore is incorporated in all forensic prediction models to date, as the most informative age marker. The possible underlying reason is that the DNA methylation levels in the specific locus of ELOVL2 are relatively stable between different samples (Sukawutthiya et al., 2021).

In the present study, there is an excellent positive statistically highly significant correlation between DNAm status at studied CPG(s) sites of ELOVL2 gene and predicted age. These result in accordance with Garagnani et al. (2012) who stated that the methylation level of CPG sites in the ELOVL2 promoter strongly correlates with age and the change in methylation level with aging is significant, spanning from 7% to 91%. In contrast with Zbieć-Piekarska et al. (2015) who stated that CPG sites 5 & 7 were found to be most significantly correlated with age and Johansson et al. (2013) who reported that the strongest positive correlation of methylation with age is seen in a CPG_1 in the promoter of ELOVL2.

5. CONCLUSION

Based on our satisfactory and promising results regarding the correlation of methylation patterns and chronological age, this study suggests that methylation of ELOVL2 can be used as an indicator of age prediction. This advances in technology and molecular biology can be used as very important tools in forensic practice.

6. RECOMMENDATIONS

- It is important to mention that the process of age prediction models by DNA methylation requires techniques and equipment with which forensic laboratories do not usually count and / or perform on a daily basis, which implies additional costs and adequate staff training.
- Another important condition that must be taken into account is that since methylation is an epigenetic mechanism that regulates gene expression, age prediction analysis cannot be based completely on the results obtained from previous studies, since the population groups are exposed to various environmental factors that could change gene regulation and therefore methylation patterns. Each population group must be sequenced and analyzed to standardize not only the technique, but also the reference parameters used for the results interpretation. Further study is necessary to improve the prediction accuracy for older age categories

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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الملخص العربي

مثيلية الحمض النووى الديوكسي ريبوزى كوسيلة للتنبؤ بالعمر

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مثيلة الحمض النووي (DNAm) هو تعديل كيميائي حيوي يحدث على مدى عمر الفرد وهو مكون مهم في عملية الشيخوخة. درجة مثيلة الحمض النووي مرتبطة بشكل كبير بالعمر. الجين ELOVL2 هو أكثر علامات العمر تقييمًا بدقة حتى الآن. وقد ثبت أن هذا الجين يمكن تحليله بشكل موثوق في بقع الدم البشرية القديمة والحديثة ، والتي تعد مصدرًا رئيسيًا للحمض النووي في مختبرات الطب الشرعي. هدف العمل: هدفت الدراسة الحالية إلى تقييم استخدام مثيلة الحمض النووي على جين ELOVL2 المأخوذ من عينات الدم للمصريين كمؤشرات حيوية لتقدير العمر الزمني باستخدام التسلسل الحراري. النتائج تم تحليل 80 عينة دم كاملة من الأفراد الذين تتراوح أعمارهم بين 18-96 سنة مقسمة إلى 4 مجموعات باستخدام مقايسة مثيلة الحمض النووي على أساس تحويل ثنائي الكبريتيت والتسلسل الحراري للحمض النووي في 7 مواقع CpG في ELOVL2. تُظهر نتائجنا ارتباطًا قويًا بين الحمض النووي والعمر الزمني ، وهو النموذج الذي يدعم الحمض النووي كمتنبئ قوي بالعمر. كانت دقة التنبؤ بالعمر أفضل في الفئة العمرية الثالثة (40-49 سنة) وكانت أقل دقة في الفئة العمرية الرابعة للأفراد المسنين (50-69 سنة) في اختيار الفرق 1 و 2.5 و 5 سنوات.