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RESEARCH

APPLICATION OF DNA METHYLATION ON URINE SAMPLE FOR AGE ESTIMATION

(Penggunaan Metilasi DNA dalam Perkiraan Umur Individu di Sampel Air Kemih)

Rosalinda Avia Eryatma[1], Puspa Wardhani[2], Ahmad Yudianto[3]

ABSTRAK

Perkiraan umur sangat penting dalam analisis forensik. Umur individu lebih sering diperkirakan dengan menggunakan tulang dan gigi. Namun, hanya terbatas pada kasus tertentu yang berhubungan dengan kerangka manusia. Metilasi DNA merupakan salah satu cara dalam memperkirakan umur di sampel biologis. Perkiraan umur menggunakan metode metilasi DNA dengan penggunaan sampel air kemih hingga saat ini belum pernah ada yang melakukan, oleh sebab itu penelitian ini akan mengunakan isolasi metilasi DNA dalam memperkirakan umur individu di sampel airkemih. Di penelitian ini digunakan 6 sampel air kemih yang didapatkan dari pendonor sehat. Tahap pertama adalah isolasi DNA dengan menggunakan DNAzol dan kloroform setelah itu, dikonversi bisulfit dengan kit DNA metilasi. Hasil isolasi kemudian di amplifikasi dengan metode PCR dan di elektroforesis dengan gel agarosa. Hasil elektroforesis dapat sebagai acuan panjang pita yang di sekuensing. Hasil sekuensing dianalisis persen metilasinya dan korelasinya dengan perkiraan umur. Hasil dari pembacaan aplikasi dan perhitungan tersebut di sampel 001 menunjukkan 64,99%, sampel 002 menunjukkan 69,45%, sampel 003 menunjukkan 57,52%, sampel 4 menunjukkan 58,61%, sampel 005 menunjukkan 63,66% dan sampel 006 menunjukkan 61,19%. Berdasarkan hasil penelitian ini merupakan langkah awal dalam penggunaan metilasi DNA dalam perkiraan umur individu terutama di sampel air kemih.

Kata kunci: *Perkiraan umur, metilasi DNA, konversi, bisulfit, sekuensing, air kemih*

ABSTRACT

Age estimation is very important in forensic analysis. Age is more often estimated using bones and teeth. However, it is only limited to specific cases related to the human skeleton. DNA methylation is one way of estimating the age of biological sample. Nevertheless, age estimation using DNA methylation method with the use of a urine sample still has never been conducted until now. Therefore, this research aimed to analyze how the isolation of DNA methylation in a urine sample is used to estimate individual age. This research used six urine samples obtained from healthy donors. The first stage conducted was the isolation of DNA using chloroform DNAzol. Second, bisulfite was converted DNA methylation kit. Third, the isolation results were amplified by PCR and then electrophoresed using agarose gel. Fourth, the electrophoresis results were used as the reference length of ribbon in sequencing. Fifth, the sequencing results were analyzed for its methylation percentage and its correlation with age. Sixth, results from the reading of the application and the calculation of sample 001 showed 64.99%, 69.45% for sample 002, 57.52% for sample 003, 58.61% for sample 004, 63.66% for sample 005, and 61.19% for sample 006. Finally, the results of this research can be used as the first step in the use of DNA methylation for individual age estimation, especially using urine sample.

Key words: Age estimation, DNA methylation, bisulfite, sequencing, urine

INTRODUCTION

Age estimation is very important in forensic analysis. Human age is often estimated using bones and teeth. However, it is limited only in certain cases related to the human skeleton. Thus, another method of estimating the age of a biological sample is needed. Nevertheless, age estimation method using DNA still has not been available. DNA methylation is one way of estimating the age of a biological sample. Biological

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sample can be obtained from urine samples. Urine is a product produced by kidneys through excretion system. Urine is composed of water, urea, ammonia, proteins and epithelial cells.[1–3]

DNA methylation is an epigenetic modification that is the best way to estimate the age of a human biological sample. This is because human DNA also experiences aging process as arranged in a process controlled by special epigenetic modification. However, this replication modification process is only found at position 5 of the pyrimidine ring of cytosine in CpG dinucleotide sequences.[2,3]

Age estimation using DNA methylation method actually has previously been carried out by researchers in 2014. Those researches used a sample of blood to isolate and identify DNA. The results of those researches showed that DNA methylation in blood sample can be used to predict the age of an individual. Nevertheless, age estimation using DNA methylation method with the use of a urine sample still has never been performed. Therefore, this research aimed to analyze how the isolation of DNA methylation in a urine sample can be used to estimate the age of an individual.[3]

METHODS

Urine sample used in this research was obtained from healthy donors in the area of Surabaya under the approval of the ethical code applied and the consent of the donors. The number of samples used was six samples with three categories of age range. The first category was the category of children aged 5–11 years old. The second category was the category of teenagers aged 12–25 years old. And, the third category was the category of adults aged 26–45 years old. However, samples used only one of the age ranges for each sex. Consequently, the number of samples used was six samples. The use of those six samples was expected to represent the results of the use of DNA methylation in urine sample.[4]

Next, urine as much as ±15 mL was put into a 15 mL centrifuge tube. The tube containing urine sample then was centrifuged with a speed of 6000 rpm for 20 minutes at –20°C. The supernatant was used in the next step, namely isolation of DNA methylation.

DNA from urine samples was isolated using phenol with a brand DNAzol® (Invitrogen Tech-Linesm) and chloroform. Next, it was isolated using DNA methylation kit from *EZ DNA Methylation-Direct™* (Zymo Research Corp., CA). DNA isolated using chloroform DNAzol then was converted with bisulfite in accordance with the instructions attached to the kit. Finally, PCR was conducted on the results of DNA elution. For negative control, *aquadest* was used, while for positive control, K562 was used.[5]

Primers used in the PCR amplification process were *T7 Promoter tag* as a *reverse primer* with the sequence 5'-CAGTAATACGACTCACTATAGGGAGAAG GCT-3 'and 10 mer tag as a *forward primer* with the sequence 5'-AGGAAGAGAG-3'. In conducting the PCR amplification, *HotStarTaq Plus Master Mix* Kit from Qiagen, USA was used.[6]

In the optimization cycle, activation was performed on a *Thermal Cycler* with a temperature of 95°C for 5 minutes. Next, the other three cycles, namely denaturation, *annealing,* and extension were performed. The denaturation stage was carried out at 94°C for 1 minute, *annealing* stage at a temperature of 68°C for 1 minute, and then extension stage at 72°C for 1 minute.[6]

Those cycles were repeated as many as 35 cycles, and then the samples were incubated at 72°C for 10 minutes. After that, they were left overnight at –20°C. The same treatment was conducted in the positive and negative controls. At the end, PCR results can be viewed on a 1% agarose gel electrophoresis.[6]

Samples of the PCR product needed were as much as 30 mL to identify CpG sites using *Applied Biosystems 3130 XL Genetic Analyzers* to measure the isolated methylation status of methylation age marker on the donors. Primer used was T7 with the sequence 5'-CA GTAATACGACTCACTATAGGGAGAAGGCT-3 '. Finally, results of the sequencing then was read with the bioedit®.[7]

The sequencing results obtained were in the form of CpG island. CpG island can be viewed and analyzed using an online application, *Emboss Cpgplot* (on site http://www.ebi.ac.uk/Tools/seqstats/emboss_ cpgplot/). Percentage of methylation can be calculated using the following formula:

$$\%\ \text{Menthylation} = \frac{\textit{length estimation of CpG}}{\textit{number of sequence}} \times 100\%$$

Next, the results obtained were regressed between age and methylation percentage presented in the DNA of the individual. To determine how much methylation percentage affects age estimation, a statistical test was performed using linier regression test.[3,8]

RESULTS AND DISCUSSION

To examine whether there was DNA in isolation and PCR results or not, visualization was conducted

using agarose gel electrophoresis. Figure 1 showed the results of agarose gel electrophoresis with isolation stages using phenol and chloroform. Afterwards, the isolation results were converted in bisulfite conversion using methylation DNA kits of *EZ DNA Methylation-Direct™* (Zymo Research Corp., CA). The isolation results then were in PCR using reagents from *HotStarTaq Plus Master Mix* (Qiagen, USA). There was a band or tape on the six samples. The dominant one appeared in the tape lengths of 150 bp and 300 bp. There was also an allele DNA band indicating DNA specifications of the donors.

Based on these results, DNA in the urine samples of the donors was successfully isolated, and then in the bisulfite conversion and PCR. The next stage of age estimation in the urine samples was sequencing. It aimed to determine the percentage of methylation in each DNA sample. Sequencing was performed at the approximate length of 300 bp band. The sequencing results then were analyzed to correlate methylation percentage with each donor's age.

The results were analyzed using an online application program, *Emboss CpGplot* (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/). The results generated indicated the emergence of the approximate area, *CpG islands*. Examples of the Emboss CpGplot results can be seen in Figure 2. This figure depicts the results of sample 001 with the category of 9 years old male children. The results of the reading were then converted into percentage.

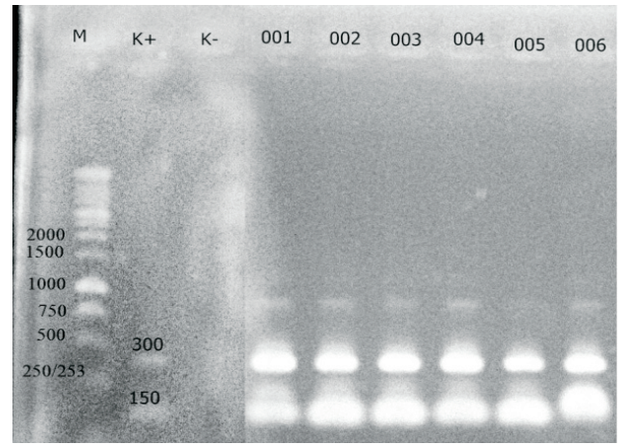

Figure 1. Results of agarose gel electrophoresis.

Note:
M = DNA marker (1 Kb)
K + = positive control (K562)
K- = Negative control (Aqudest)
001 = urine sample category of 9 years old male children
002 = urine sample category of 9 years old female children
003 = urine sample category of 17 years old male teenagers
004 = urine sample category of 17 years old female teenagers
005 = urine sample category of 31 years old male adults
006 = urine sample category of 39 years old female adults

A formula used to calculate sample 001 with the category of 9 years old male children was as follow: The approximate number of CpG on sample 001 according to the Emboss CpGplot application was 271. Meanwhile, the number of sequences derived from the sequencing results was 417.

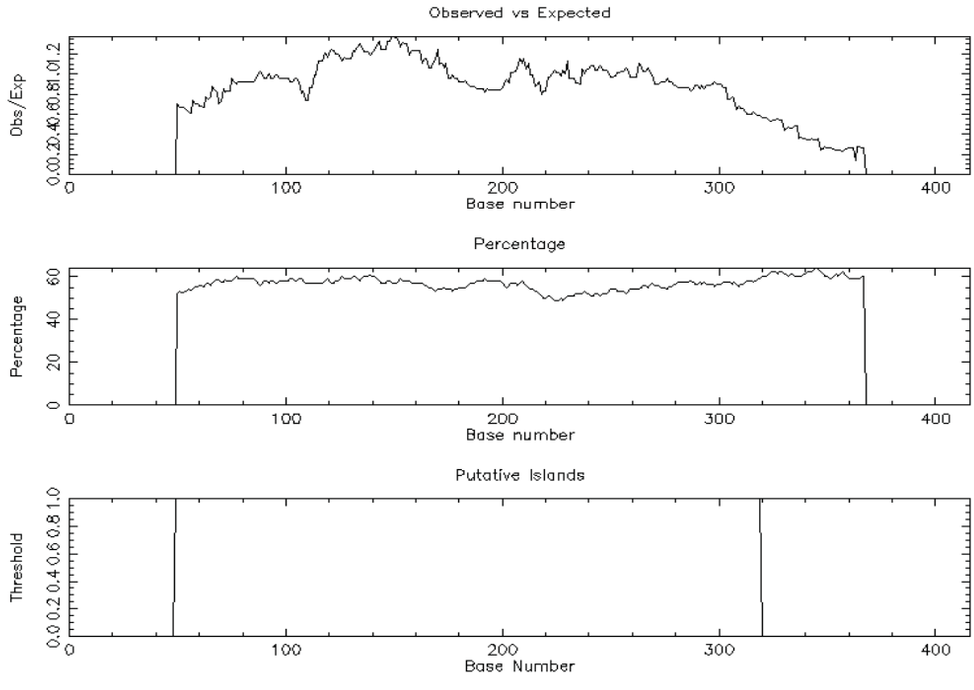

Figure 2. Analysis results of CpG islands with *Cpgplot Emboss* application program.

Table 1. Calculations results of methylation percentage at a length of 500 bp

Code	Age (years)	Sex	Estimated length of CpG site	Number of sequence	Methylation percentage (%)
001	9	Male	271	417	64.99
002	9	Female	291	419	69.45
003	17	Male	241	419	57.52
004	17	Female	245	418	58.61
005	31	Male	261	410	63.66
006	39	Female	257	420	61.19

Methylation sample 001 = $\frac{271}{417} x\ 100\%$

Methylation sample 001 = 64.99%

CPGPLOT islands of unusual CG composition
2143664_003A_P_reverse from 1 to 417
Observed/Expected ratio > 0.60
Percent C + Percent G > 50.00
Length > 200
Length 271 (50..320)
Created emboss_cpgplot-E20160130-135456-0496-59527734-oy.1.png

With the use of the same application and the same calculation formula as those for sample 001, the percentage of methylation in those six samples can be seen in Table 1.

After the calculation was conducted, those six samples were analyzed using graphs. The purpose of the graph was to see the regression between the percentage of methylation and the age of the sample donors. This correlation graph can be seen in Figure 3.

The correlation results obtained from the graph was a non-linear graph. It meant that the percentage of methylation in individual was various. The results obtained, however, still did not prove the existence of a linear correlation between individual age and methylation percentage in accordance with the purpose of this research. Consequently, to determine the influence of methylation percentage on age estimation, linear regression test was performed. The test results obtained show that the linear correlation value was 74.9% (R=0.749). This means that there was a correlation between methylation percentage and age estimation, about 74.9%.

Based on the linear regression test, moreover, a parameter value generated was 0.320. The value of this parameter was useful to analyze the correlation of estimated methylation percentage and age estimation. Calculation to age estimation was *parameter x estimated percentage*. The results of the calculation were shown in Table 2. The results of the calculation then was applied to a graph as shown in Figure 4.

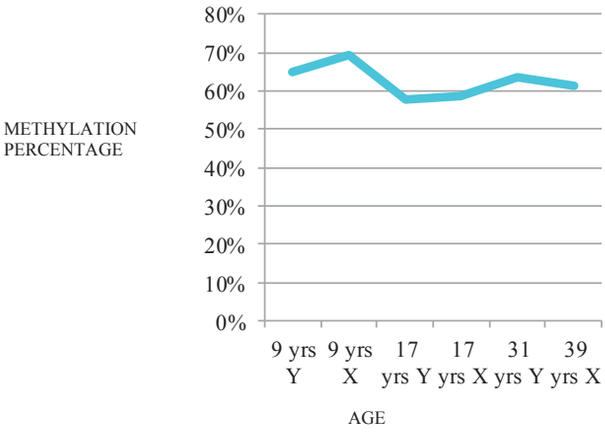

Figure 3. The correlation graph between age estimation and methylation percenmtage.

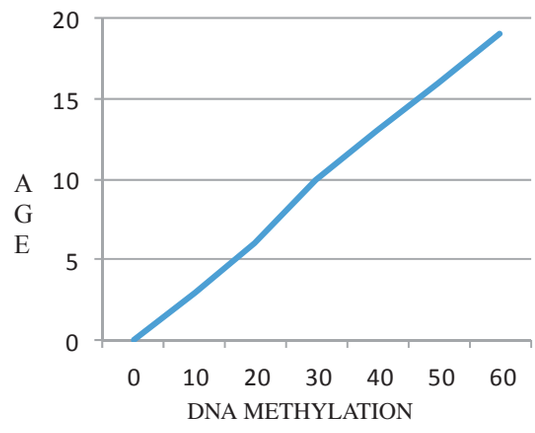

Figure 4. The correlation graph between age estimation and DNA methylation.

Note:
X= Females
Y= Males

Table 2. Table of age estimation

Percentage (%)	Parameter	Age estimation (years)
0	0.320	0
10	0.320	3
20	0.320	6
30	0.320	10
40	0.320	13
50	0.320	16
60	0.320	19

There were several possibilities as to the non-linier results of methylation percentage. Technically, there may be a lack of precision of the researchers in the bisulfite conversion. The lack of precision in the bisulfite conversion might result in some clusters C that were not converting entirely, so the levels of methylation percentage can increase.[9]

Based on some literatures, small levels of DNA in this research can affect methylation percentage. DNA levels in urine samples actually are the highest ones among DNA levels in other biological samples. For instance, blood serum has the highest level of DNA among other biological samples.[9]

Finally, the non-technical influencing factor is derived from donors, thus, affecting the quality of the donors' urine sample. Besides the increasing of age, poor nutrition, lifestyle and environmental factors, such as smoking could also affect the quality of the urine samples. These factors then can make the DNA in the body not converting entirely at shown in the methylation percentage of the urine samples studied. Therefore, further research is needed to determine methylation percentage and its correlation with individual age.[10]

CONCLUSION AND SUGGESTION

Based on the results and discussion, it can be concluded that the results of this research can be considered as the first step in isolating and converting bisulfite in urine samples. However, the results still have not been able to prove how methylation percentage can estimate the age of an individual since the correlation value obtained was only 74.9% (R=0.749). Thus, the results of the research were still not efficient in determining the correlation between age estimation and DNA methylation, especially in urine samples. Consequently, further researches are needed to learn more about the correlation between age estimation and DNA methylation. Finally, sample variations and sample size may be considered in future researches, especially in urine sample.

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