Human Age Estimation Through DNA Metilation Analysis Method from Bite Mark Samples in Forensic Identification

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Human Age Estimation Through DNA Metilation Analysis Method from Bite Mark Samples in Forensic Identification

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Abstract

Background: Age estimation are often calculated using bone and teeth samples, but are limited to human skeleton findings. The aim of this research is to estimate human age through DNA methylation method from bite mark samples. **Result:** There were 40 bite mark samples obtained from healthy volunteers. DNA samples were isolated using DNAzol reagent and converted using DNA methylation kit. The isolated DNA were amplified and electrophoreses was conducted using agarose gel. Electrophoresis result was used as length reference for the sequenced band and analyzed for methylation percentage and correlation with age estimation. Statistical test showed that there was a significant correlation between DNA methylation percentage with age estimation both in men (r Pearson 0.767) and women (r Pearson 0.878). **Conclusion:** Cpgplot emboss analysis for DNA methylation mean percentage in men tend to increase in accordance to age categories, whereas for women, the mean DNA methylation percentage in age categories was stable or constant.

Keywords: *Age estimation, DNA methylation, Bite mark*

Background

Forensic identification helps investigators determining a person's identity. Personal identification is often a challenge in both criminal and civil cases. Determining personal identity appropriately is very important in investigations because errors might bring fatal consequences in judicial process.[1]

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Forensic medicine plays a crucial role in identification, especially for unidentified corpses, damaged corpses, decomposed corpses, burnt corpses and corpses that were involved in mass accidents, natural disasters, riots that resulted in many deaths, as identification for pieces of human corpses or skeletons or others. Forensic identification also plays a role in various other cases such as kidnapping of children, swapped babies, or babies with questionable parents. A person identity can be confirmed if at least two methods give positive results (no doubt).[2]

In certain cases, for example sexual assault, bite marks are often found. The presence of physical evidence such as bite marks in rape, murder and violence cases is crucial. Bite marks are the most common evidence in rape cases. This evidence also plays a role to determine the physical violence type and the offender's age.[3,4]

Suspect identification process using bite marks as evidence is generally carried out by comparing photo interpretation with teeth model of the suspected offender, including analysis and measurement of size, shape and position of each tooth.[5] However, this technique does not always bring accurate results, therefore another way to identify bite marks is conducted through irrigation technique on bite marks to conduct DNA identification. DNA samples from bite marks could be taken from saliva, attached stains, mucosal epithelial remains in saliva, and others.[6]

Age estimation using biomolecular approach can be carried out based on analysis of cell, mitochondrial DNA (mtDNA) sequences' changes and epigenetic changes, which do not affect DNA.[7] Oral cavity as well as teeth tissues have various cells that could be used as genetic material source. Several methods that might be used to determine chronological age from teeth samples include aspartic acid racemization (AAR), mitochondrial DNA mutations, telomere shortening, advanced glycation end-products (AGEs), and DNA methylation.[8,9,10] DNA methylation is a modification process of 5 'carbon atoms in cytosine residue followed by guanine residue, therefore it is called as CpG (s)/CpGsites dinucleotides. Replication process in DNA methylation is only found at 5 pyrimidine rings position from cytosine in CpG dinucleotide sequence.[7,11] DNA methylation is the best epigenetic modification method for estimating age from human biological samples. The purpose of this study was to determine age estimation through DNA methylation analysis from bite mark samples [12,13,14]

Methods

Methods and samples

This study was an analytic cross-sectional design observational study. The samples in this study were bite marks on glasses used by 40 volunteers who have assigned their informed consent, consisting 20 men and 20 women ranging ages from 5 to 65 years. This study has received ethical eligibility from Faculty of Dentistry which number: 350 / HRECC.FODM / VII / 2020.

Sample preparation

Figure 1. Volunteer drinking glasses

A total of 30 bite mark samples were stored in conical tubes/dinking glasses (figure.1) and were labeled with 1-40 (sample number's order)-XY/XX (sex)-category (according to WHO). Human age categories according to WHO (Depker RI, 2009) is divided into four groups: children (5 - 11 years), adolescents (12-25 years), adults (26-45 years) and elderly (46-65 years).

DNA extraction

Table 1. Measurement of Sample Consentration and Purity DNA

Criteria	Age (Years Old)	Sex	Consentration DNA bite marks (ng/µl)	Purity DNA bite marks
Children	5.0	L	31.45	1.20
	5.4	L	37.56	1.32
	6.3	P	45.34	1.45
	6.7	L	56.25	1.09
	7.0	P	56.15	1.34
	7.7	P	39.36	1.21
	7.8	L	78.65	1.35

***Cont...* Table 1. Measurement of Sample Consentration and Purity DNA**

	8.2	L	89.35	1.46
	10.2	P	56.57	1.57
	10.8	P	35.37	1.08
Youth	12.3	L	45.38	1.12
	13.4	L	67.56	1.22
	13.7	P	68.74	1.34
	13.7	P	78.64	1.54
	15.0	P	76.64	1.65
	16.7	L	56.45	1.23
	17.0	P	35.56	1.21
	17.0	P	32.65	1.02
	18.0	L	33.35	1.22
	22.0	L	43.47	1.21
Adult	26.5	L	42.35	1.33
	27.0	L	34.45	1.31
	27.6	P	56.65	1.21
	28.0	P	65.34	1.31
	30.5	P	54.21	1.32
	32.0	P	54.15	1.44
	37.2	L	55.25	1.03
	40.1	L	67.54	1.20
	43.0	P	78.42	1.32
	44.2	L	98.23	1.33
Ederly	47.0	L	78.43	1.21
	47.4	L	88.56	1.02
	48.0	L	67.75	1.21
	49.0	P	77.43	1.11
	50.0	P	65.17	1.07
	51.0	L	55.31	1.43
	51.3	L	45.65	1.23
	57.0	P	34.32	1.21
	58.1	P	47.14	1.54
	65.0	P	54.54	1.32
	Average		55.78	1.27

Isolation / extraction was carried out using DNAzol reagent. Samples in pellet form inside conical tubes were added 1 ml of DNAzol reagent, both were mixed by vortex, then incubated for 5 minutes at room temperature. Afterwards, 10,000 rpm centrifuge was carried out for 10 minutes at 4^0C, the viscous supernatant was taken and transferred into a new tube. The new tube is added 0.5 ml of 100% ethanol (absolute), mixed, and incubated at room temperature for 1-3 minutes, and then centrifuged at 4000 rpm speed for 1-2 minutes at 4^0C. Supernatant was removed carefully to prevent accidental removal of DNA (pellets). The pellets were washed with 0.8-1 ml of 75% ethanol 2 times and each time the tube was washed back and forth for 3-6 times. The tube was placed in an upright position for 0.5 - 1 minute, then 75% ethanol was removed by pipetting or decanting. The pellets were dried by leaving the tube open for 5–15 seconds. The pellets containing DNA were dissolved with 25-30 ul of distilled water, and then were vortexed adequately, and then stored at -20^0C. Furthermore, DNA level and purity measurement were carried out using a UV Spectrophotometer, the results were mean DNA level and purity, as in table 1 and figure 2

Bisulfite conversion (*MethiylEdge*^{TM} *Bisulfite*)

Fifty microliter of DNA samples in eppendorf tube were added with 130 µl of CT Conversion Reagent. This mixture was then centrifuge at 5000xg speed for 1 minute. PCR tube was placed in PCR machine with the following setting: 980C for 8 minutes, 640C for 3.5 hours, and kept in a storage at 40C for up to 20 hours. Furthermore, 600 µl of M-Binding Buffer was poured into Spin IC column with samples from PCR results. The column was closed and stirred by turning the column several times. Afterwards, it was centrifuged at 10000xg speed for 30 seconds, then the supernatant was removed. The resulting pellets were washed with 100µl M of wash buffer into the column, and then centrifuged at 10,000xg speed for 30 seconds. The supernatan was removed, and the pellets were added with 200µl M of Desulphonation Buffer, and then incubated at 200C for 20 minutes. Centrifuged was carried out again at 10,000xg speed for 30 seconds. Pellets were then washed twice with 200µl M of Wash Buffer and then centrifuged at 10000xg speed for 30 seconds. Finally, 10µl M of Elution Buffer was added and centrifuge was carried out at 10000xg speed for 30 seconds.

PCR amplification

PCR amplification was carried out using T7 promoter tag primer and 10 mer tags and HotStarTag Plus Master mix kit. The setting was as followed: temperature of 950C for 5 minutes in 30 cycles (denaturation: temperature of 940C for 1 minute, annealing: temperature of 650 C for 1 minute, extension: temperature of 720C for 1 minute), temperature of 720C for 10 minutes. PCR result using 1% agarose gel electrophoresis showed bands between 150pb-300pb (figure.3).

Sequencing

Table 2. Percentage of DNA methylation in bite marks samples at a length of 300 bp

Criteria	Age (Year Old)	Sex	Length of CpG sites	Number of sequens	Methylation Percetage (%)
Children	5.0	L	123	298	41,28
	5.4	L	109	278	39,21
	6.3	P	210	298	70,47
	6.7	L	121	278	43,52
	7.0	P	89	296	30.07
	7.7	P	191	296	64,53
	7.8	L	176	289	60,89
	8.2	L	134	279	48,03

Cont... **Table 2. Percentage of DNA methylation in bite marks samples at a length of 300 bp**

	10.2	P	98	288	34,03
	10.8	P	126	298	42,28
Youth	12.3	L	117	299	39,13
	13.4	L	110	298	36,91
	13.7	P	111	288	38,54
	13.7	P	99	286	34,62
	15.0	P	161	276	58,33
	16.7	L	76	289	26,29
	17.0	P	134	279	48,03
	17.0	P	98	298	32,88
	18.0	L	120	300	40,00
	22.0	L	126	299	42,14
Adult	26.5	L	121	296	40,88
	27.0	L	111	297	37,37
	27.6	P	129	289	44,64
	28.0	P	121	278	43,53
	30.5	P	126	294	42,86
	32.0	P	114	286	39,86
	37.2	L	118	292	40,41
	40.1	L	120	300	40,00
	43.0	P	126	299	42,14
	44.2	L	121	296	40,88
Ederly	47.0	L	111	297	37,37
	47.4	L	129	289	44,64
	48.0	L	121	278	43,52
	49.0	P	121	296	40,88
	50.0	P	111	297	37,37
	51.0	L	120	300	40,00
	51.3	L	126	299	42,14
	57.0	P	120	300	40,00
	58.1	P	126	299	42,14
	65.0	P	125	267	46,82

The sequencing process required 30 µl of PCR sample to identify CpG sites as methylation age marker. The primer used was T7 with 5-CAGTAATACGACTCACTATAGGGAGAAGGCT-3' order. The results of sequencing with Applied Biosystems 3130 XL Genetic Analyzers were read with bioedit®. In FASTA form, CpG sites percentage was calculated using an online application, Emboss Cpgplot

(http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) (Table.2)(figure.4).

Results

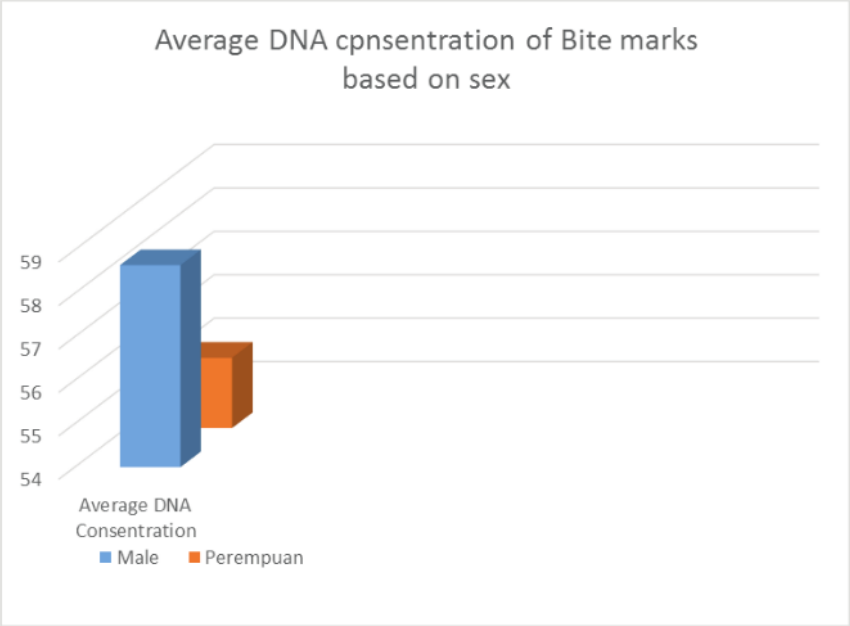

Figure 2. Graph of average DNA consentration in bite marks sample

Figure 2 above shows that average DNA consentration sample from bite marks was 55.78 ng/µl, while DNA purity ranged from 1.02 to 1.65. Electrophoretic DNA visualization with 1% agarose gel revealed DNA bands ranging from 150bp to 300bp.

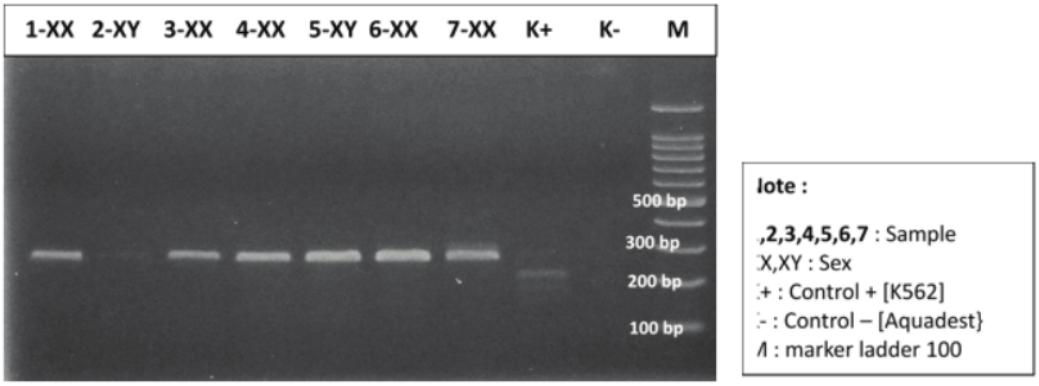

Figure 3. PCR visualization of bisulfite converted DNA bite marks samples

Figure 3 above shows the results of agarose gel electrophoresis from bite marks samples through DNAzol isolation. Afterwards, isolated DNA were conducted bisulfite conversion using DNA methylation kit from MethiylEdge TM Bisulfite promega. Isolated DNA samples were then analyzed in PCR machine using reagents from HotStarTaq Plus Master Mix with T7 promoter tag primers and 10 mer tags. All samples have bands / bands in 300bp core sequence.

All visualized samples were in 300bp core sequence, therefore sequencing was carried out in 300bp core sequence usong Applied Biosystems 3130 XL

Genetic Analyzers that was read with bioedit® and analyzed on CpG sites using Emboss Cpgplot application. DNA methylation percentage from bite marks samples are presented in table below.

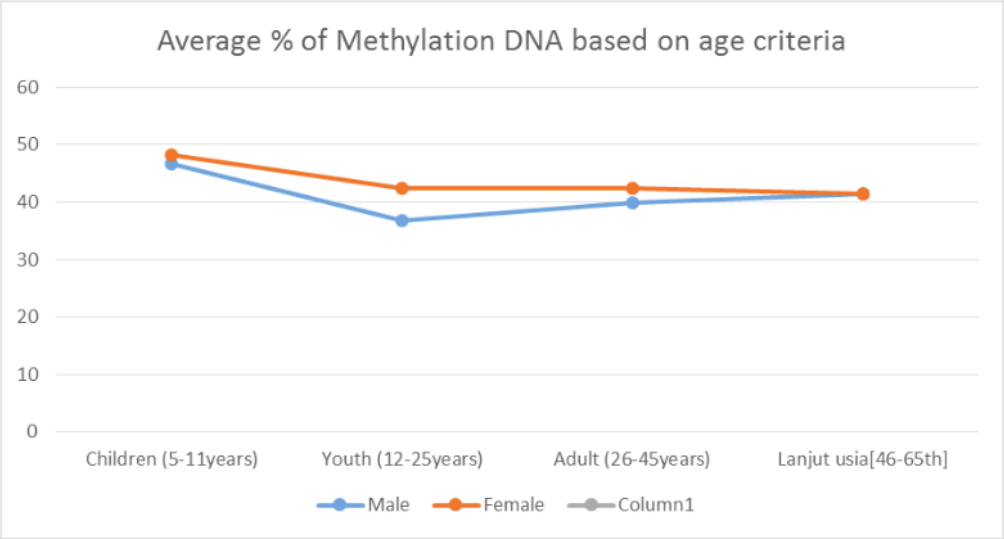

Figure 4. Graph of average percentage of DNA methylation based on age criteria

Figure 4 shows average DNA methylation percentage in men tends to increase in accordance to age categories, whereas for women the DNA methylation percentage is stable or constant. This is most likely to be influenced by lifestyle, environmental, and previous disease factors.

Discussion

In genome, methylation occurs in a CpG-rich region named CpG island.[11] Protein and methyl-CpGbonding domains will bind specifically to CpG island, inducing repression and transcriptional activation.[15]

As humans get older, DNA methylation pattern gradually shows DNA hypomethylation and hypermethylation on a specific CpG island-associated promoter.[11,15] Correlation between DNA methylation and aging can be described in two parts, which are epigenetic drift and epigenetic clock. Epigenetic drift shows a variety of changes in individuals that are associated with age due to environmental factors, on the other hand, epigenetic clock is only markedly related to age, therefor it might be used to predict an individual chronological age.[10,15,16]

Between male and female, CpG site has different DNA methylation rates. CpG site is located at X chromosome, therefore differences in sex-specific methylation at X chromosome tend to be more unstable. Women susceptibility to stress and certain diseases also greatly influences DNA methylation rate, thereby factors that influence DNA methylation are widely variable between individuals.[17,18] Degenerative diseases and metabolic syndrome of each individual also greatly affect DNA methylation percentage. The course of degenerative disease targets cellular epigenetic machine elements, altering epigenetic machines expression and activity therefore inducing epigenetic changes in each individual.[16,19]

DNA methylation process might turn into a dynamic process, where individual hormonal state greatly affects cytosine hydroxymethylation role. The use of certain oxidative and antioxidant drugs will also affect histone modification rate. This might occur, for example, via cytosine mutations in case of methylation on normal cells. Methylated cytosine absence might lead to permissive histone modification and allow genes to be expressed. Unlike genetic mutations, epimutation does not alter DNA basic sequence and is potentially reversible.[17] Certain diseases such as neoplastic, degenerative, metabolic, and inflammatory might cause oxidative stress that affects gene activation, such as

epigenetic-DNA methylation.[15]

Conclusion

Mean DNA level from bite marks was 55.78ng/µL. There was a significant correlation between DNA methylation percentage (CpG sites) from bite mark samples with human age estimation both in men and women.

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