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Forensic age prediction for dead or living samples by use of methylation-sensitive high resolution melting

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ABSTRACT

Age prediction with epigenetic information is now edging closer to practical use in forensic community. Many age-related CpG (AR-CpG) sites have proven useful in predicting age in pyrosequencing or DNA chip analyses. In this study, a wide range methylation status in the *ELOVL2* and *FHL2* promoter regions were detected with methylation-sensitive high resolution melting (MS-HRM) in a labor-, time-, and cost-effective manner. Non-linear-distributions of methylation status and chronological age were newly fitted to the logistic curve. Notably, these distributions were revealed to be similar in 22 living blood samples and 52 dead blood samples. Therefore, the difference of methylation status between living and dead samples suggested to be ignorable by MS-HRM. Additionally, the information from *ELOVL2* and *FHL2* were integrated into a logistic curve fitting model to develop a final predictive model through the multivariate linear regression of logit-linked methylation rates and chronological age with adjusted $R^2 = 0.83$. Mean absolute deviation (MAD) was 7.44 for 74 training set and 7.71 for 30 additional independent test set, indicating that the final predicting model is accurate. This suggests that our MS-HRM-based method has great potential in predicting actual forensic age.

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1. Introduction

Although age is one of the most important pieces of information for criminal investigations, there are few techniques available to predict age in actual practice, such as examining bones or teeth morphologically. These techniques require expert medical experience, but the result of prediction might not be "objective". Moreover, these are not versatile methods and are limited to samples such as bones or teeth in actual practice.

Age-related changes in cytosine methylation have been recently reported by many groups [1–7]. For example, Hannum et al. built a predictive model of aging blood with the use of 71 methylation markers selected from the Illumina Infinium HumanMethylation450 BeadChip, which measures more than 450,000 CpG markers [8]. Branicki et al. investigated the usefulness of CpGs located in the promoter region of *ELOVL2* with pyrosequencing [9,10]. The promoter region of *FHL2* has also been identified as a useful age-predictive marker in many studies [4,10]. Owing to these studies, knowledge on the relationships between methylation patterns and chronological age has accumulated. However, the BeadChip method requires specialized instruments and analyzing machines followed by complex bioinformatic analysis for age prediction. The pyrosequencing method also requires specialized instruments. In general, very few forensic laboratories are equipped with these kinds of machines. Even if so, high costs has prevented these methods from being routinely used in criminal investigations.

Methylation-sensitive high resolution melting (MS-HRM) is a method that measures methylation statuses easily, quickly and cost effectively, where bisulfite-treated DNA is PCR amplified followed by melting analysis [11–15]. In bisulfite-treated DNA analyses, unmethylated cytosines are converted to uracil by bisulfite conversion while methylated cytosines are kept intact. Therefore, the information of methylation status is directly converted to the sequence, where it alters the thermodynamic stability of double-stranded DNA, enabling quantitative methylation assessment. The unique characteristic of MS-HRM is that it measures the overall methylation status of amplified PCR products, rather than the individual CpG marker. As a result, the information of many CpG markers present in the region of interest can be integrated and analyzed with one pair of PCR primers in one measurement.

Abbreviations: MS-HRM, methylation-sensitive high resolution melting; MAD, mean absolute deviation.

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We have to consider the possibility that post-mortem changes alter the methylation status when performing age prediction in actual cases. For example, a forensic scientist is not always cognizant of whether a victim is alive or deceased, as in abduction cases. Before applying this technology to actual cases, we must investigate the effect of post-mortem changes on forensic age prediction. To our best knowledge, no one has focused on this point, which might become a more significant issue when performing age prediction for actual forensic cases.

Here, we report on a labor-, time-, and cost-effective method of forensic age prediction using MS-HRM for the *ELOVL2* and *FHL2*. The analysis of 74 blood samples from 22 living and 52 dead donors who varied in age from 0 to 95 years yielded a logistic curve model. While the majority of previous studies constructed simple linear models for this analysis, such models were not rational for the purposes of our study. Finally, 30 independent dead blood samples were used to test the prediction accuracy of the model.

2. Materials and methods

2.1. Sample collection and DNA extraction

Blood samples from 19 healthy donors were collected at the same time of health checking. Blood samples from three children were collected from epistaxis caused by daily life hurt rather than performing any operation. For these blood samples, DNA was extracted with QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All donors or their parents signed written consent form prior to donation. Additionally, cadaver blood samples were collected from 82 autopsies performed during 2006–2009 at Kyoto University, Kyoto, Japan. Cadaver blood samples were collected in cases of extrinsic death—such as burn cases or suicides—and all autopsies were performed within 10 days of death. All dead bodies had no evidence of disease (e. g. cancer) which affects the methylation status. DNA from cadaver blood was extracted using the QIAprep DNA Blood Kit and stored at -20 °C until use. All samples in this study were used with permission for research use from the ethical committee of Graduate School of Medicine of Kyoto University.

2.2. Bisulfite modification and control DNA

All DNA extracted from blood was treated with EpiTect Fast Bisulfite Conversion Kit (Qiagen) and bisulfite-converted DNA were eluted with Buffer EB (10 mM Tris-Cl, pH 8.5). The concentration of eluted DNA was then measured with the Nano Vue Plus (GE Healthcare, Amersham, England) and subsequently adjusted to 10 ng/μL with Buffer EB. As a positive (fully methylated) or negative (fully unmethylated) control, we used "EpiTect Control DNA (human), methylated/unmethylated and bisulfite converted (Qiagen)" respectively. Control DNA was stored in Buffer EB and adjusted to 10 ng/μL.

2.3. High resolution melting step

PCR primers were designed with BiSearch [16,17] according to Table 1. For *ELOVL2*, the amplicon is 91 bp long and includes 10 CpG markers between primer binding sites (chr6: 11,044,611–11,044,701; Genome browser UCSC GRCh38, Fig. 1). For *FHL 2*, the amplicon is 133 bp long and includes 14 CpG markers (chr2: 105,399,228–105,399,360). PCR amplification was carried out with a Roche LightCycler 480 Instrument II (Roche Diagnostics GmbH, Mannheim, Germany) equipped with the Gene Scanning Software (version 1.5.1.62 SP2) in a 25 μL total volume containing: 1× EpiTect HRM PCR Master Mix (EpiTect HRM PCR Kit, Qiagen), 250 nM of each primer and 20 ng of bisulfite modified template. First, polymerase was activated at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 50 °C for 30 s, and 72 °C for 10 s. After the amplification, HRM analysis was initiated by denaturing all products at 95 °C for 1 min, followed by re-annealing at 40 °C for 1 min. Subsequently, the samples were quickly warmed to 50 °C and heated to 95 °C at 0.1 °C/s. Fluorescence intensity was measured at 25 acquisitions/s. All reactions were performed in duplicate.

Table 1
The sequence of PCR primers.

Primers	Primer sequence (5′ to 3′)
ELOVL2-Fw	CGATTTGTAGGTTTAGT
ELOVL2-Rv	ACTACCAATCTAAACAA
FHL2-Fw	TTTACCAAAACTCCTTTCTT
FHL2-Rv	GTGGGTAGATTTTTGTTATT

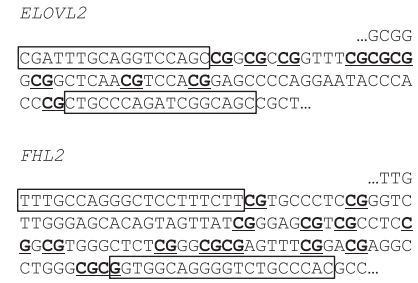

Fig. 1. Sequences of PCR target sites in this study (before bisulfite conversion). PCR primer binding sites are boxed. CpG markers that can be analyzed by MS-HRM are emphasized and underlined.

When HRM analysis was performed, Gene Scanning Software first normalized raw melt curves so that different samples can be compared. In this normalizing process, we set the pre-melt temperature region to 68–69 °C and the post-melt temperature region to 82–83 °C. Although, the temperature shift process is often run when the software is used for analyzing heterozygous mutant, no adjustment was performed in this study by setting the threshold to zero, because the shape of melt curve itself was important in analysis of the overall methylation status of the amplicon. If the temperature shift process was performed, the shape of melt curve would be distorted. A difference curve was then derived from the first derivative of the melt curves, after setting the data of fully unmethylated sample as a baseline. Relative signal difference values were exported as .txt data, and the maximum absolute value were defined as "Df value" for each sample (Fig. 2B).

2.4. Methylation analysis

In general, PCR bias occurs when amplifying bisulfite-treated DNA [18,19], since unmethylated DNA (UG pair rich sequence after bisulfite modification) tends to be amplified more efficiently than methylated DNA (CG pair rich sequence). Therefore, a standard curve was first established for each target site to accurately measure methylation status. Fully methylated control DNA and fully unmethylated DNA were mixed in appropriate ratios to make 0%, 25%, 50%, 65%, 80%, 90%, and 100% methylated control DNA. For the 90% and 100% methylated standard sample of *ELOVL2*, 40 ng bisulfite-treated DNA was used as a template due to its small amplification efficiency caused by PCR bias. Df values of each control sample were plotted and a non-linear regression model was developed [18] with R (version 3.2.2) [20] depicted as Eq. (1) as follows

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