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Jana Naue · Julia Winkelmann · Ulrike Schmidt · Sabine Lutz-Bonengel

Institute of Forensic Medicine, Medical Center—University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

Analysis of age-dependent DNA methylation changes in plucked hair samples using massive parallel sequencing

Introduction

The analysis of DNA methylation (DNAm) is an interesting forensic tool for applications, such as age prediction and tissue or body fluid determination [30]. In the past, the investigation in the field of age prediction and the collection of reference data mainly focused on blood, saliva and buccal swab samples [5, 9, 21]. In a few studies, other tissues were examined and some of the commonly used DNAm markers were found to be age-dependent in multiple tissues, of which some showed very tissue-specific changes with age [3, 13–15, 23]. Another important trace found at crime scenes is hair that has not yet been considered for age prediction using DNAm analysis. Hair is a challenging type of sample with a high variety of DNA quality and amount, and the analysis of age-dependent DNAm markers depends on the availability of nuclear DNA. Therefore, the analysis of hair is restricted to hair with an available root still containing intact hair cells. Hair and also nails are examples of keratinous tissues, with the special characteristic that the tissue originates from a living precursor cell ending in cell death during each hair cycle [4]. In forensics, the term “hair” often refers to the hair shaft only or to a hair shaft with a root without any (detailed) specification of the presence of attached cells; however, having a look at the total hair follicle, nuclear DNA can derive from multiple cell origins developed from different germ layers: undifferentiated cells,

dermal fibroblasts, melanocytes and differentiating keratinocytes in the bulb as well as cells of the inner and outer root sheath, stem cells in the bulge and fully differentiated keratinocytes (dead cells, trichocytes) [25, 27, 29]. Furthermore, a low amount of nuclear and/or very short DNA fragments were found to be present in the hair shaft [6, 12]. The type and number of cells obtained by hair plucking depends on the technique as well as the different phases of the anagen hair, which is characterized by hair growth and lasts over years [2]. The majority of epithelial structures from the hair follicle and part of the bulge containing the stem cells for keratinocyte and melanocyte production, remain attached to the plucked hair but not all of the connective tissues [11].

The aim of this proof-of-concept study was to examine if a successful analysis by massive parallel sequencing (MPS) of known age-dependent DNAm markers can be performed using plucked hairs and if any special challenges have to be considered. Furthermore, these age-dependent DNAm markers were characterized in hair in comparison to other tissues. As hair was plucked from deceased individuals, other tissue samples of the same individuals were available for analysis. Furthermore, the sequence composition of the analyzed targets was determined, as a single nucleotide polymorphism (SNP) at the 5'-cytosine-phosphate-guanine-3' (CpG) site and surrounding region may alter the DNAm, e.g. by removal of the

CpG site itself or due to a regulatory effect of the underlying sequence composition.

Material and methods

Samples and DNA extraction

Various tissue samples, including plucked head hair from deceased individuals without signs of putrefaction were collected in a previous study in Freiburg, Germany [22] and stored at –80 °C until analysis. Approval for collection and the extended use of the samples for the conducted DNAm analysis was obtained from the ethics committee of the University of Freiburg (263/10, 397/16). Plucked hairs from 49 selected individuals were analyzed covering a wide range of ages (5–88 years) and both sexes (female 22, male 27). From each individual 10 head hairs with roots were pooled for the analysis, if available. Hairs were cleansed prior to cell lysis in the case of visible stains on the hair by incubation in 500 µL DNA-free water for 5 min at 700 rpm. One sample showing a blood stain was additionally cleaned in 500 µL Buffer AL (Qiagen, Hilden, Germany) using an incubation of 2 min and 700 rpm at 37 °C. The DNA was extracted using the QiaAmp Micro Kit (Qiagen). Hair lysis was performed using 160 µL Buffer ATL (Qiagen), 20 µL 1M DTT and 20 µL proteinase K per sample with a 60 min incubation at 60 °C and 1000 rpm. Subsequent steps were performed according to manufacturer's recommendations. The DNA was quantified using the Quantifiler

Human (Thermo Fisher Scientific, TFS, Waltham, MA, USA) or PowerQuant System (Promega, Madison, WI, USA). Reactions were run in a 10- μ L volume on an AB7500 with 7500 System SDS software v2.0.5 (TFS). To compare the DNAm of other tissues (blood, buccal cells, bone, skeletal muscle, brain tissue) DNAm data that were already available for most of the individuals were taken from a previous study [23]. The DNAm of tissue samples from 10 individuals not included in the preceding study was analyzed as described in [23].

Bisulfite conversion and ssDNA quantification

Bisulfite conversion was performed with the DNA Methylation EZ Gold Kit (Zymo Research, Irvine, CA, USA) using 30 μ L of the DNA eluate in a reduced total volume of 120 μ L for the initial deamination step. The single stranded DNA (ssDNA) was eluted in 15 μ L H₂O and quantified using the Qubit ssDNA assay kit for the Qubit 2.0 fluorometer (TFS).

PCR, library preparation and massive parallel sequencing

Singleplex amplification of 10 selected age-dependent DNAm markers was performed as described in [21], including the markers *DDO*, *ELOVL2*, *F5*, *HOXC4*, *KLF14*, *MEIS1-AS3*, *NKIRAS2*, *RPA2*, *TRIM59*, *ZYG11A*. Of the converted DNA 1 μ L was added to each PCR reaction. For each individual the successfully amplified loci were pooled and the products cleaned with 1.9x magnetic beads (GE Healthcare, Little Chalfont, UK) prepared according to [26]. Of the pooled sample 3–6 μ L was added to 12.5 μ L Q5 Hot Start Ultra HiFi PCR master mix (New England Biolabs, Ipswich, MA, USA), 5 pmol of each index primer (Biomers, Ulm, Germany, based on the dual Nextera XT indexing system from Illumina [San Diego, CA, USA]), and water ad 25 μ L. The runs consisted of 95°C 1 min, 72°C 5 min; 6 cycles: 95°C 30 s, 62°C 2 min, 72°C 2 min; final elongation at 72°C for 3 min. The PCR products were cleaned twice with prepared 1.6x beads (GE healthcare). The

DNA quantity of each amplicon pool was measured using the Qubit dsDNA HS assay kit (TFS). Amplicon pools were combined in equimolar ratios. A final 10.5 pM library containing all samples was paired-end sequenced on a MiSeq using the 300 bp v2 chemistry (Illumina, San Diego, CA, USA) on a MiSeq FGx (Verogen, San Diego, CA, USA).

Data analysis

Quality control and DNAm extraction from the raw data were performed as described before [21]. The minimum threshold required was set to 600 reads as a coverage too low leads to increased stochastic variation of the obtained DNAm [17, 20] but a coverage of at least 800–1000 was anticipated. Bisulfite conversion efficiency was checked by analysis of the non-CpG (i.e. CHH and CHG) methylation levels. Further analysis and statistical analysis were performed under the open-source Anaconda Navigator environment (Anaconda Software distribution, <https://anaconda.com>) using Jupyterlab 1.2.6 and Python 3.6. Correlation analysis between age and DNAm was accomplished using Spearman's rank correlation coefficient using the package pingouin v0.3.8 [28], along with the 95% confidence interval (95% CI) and the adjusted *p*-value for multiple testing (based on the Benjamini-Hochberg false detection rate).

SNP analysis

Sequence variation was characterized for individuals from whom a successful analysis of the DNA from hair was achieved (according to [21]). The DNA extracted from blood was used for amplification covering the analyzed DNAm loci. Sanger sequencing was performed in a 5 μ L volume using the BrilliantDye terminator sequencing kit (Nimagen, Nijmegen, The Netherlands) or BigDye v1.1 Terminator kit (TFS). Cycling was conducted as described in the manufacturer's protocol of the BigDye v1.1 terminator kit (TFS). Afterwards, samples were run on a 3130xL genetic analyzer with foundation data collection software v3.0 (TFS). Data were aligned

to the genomic references (GRCh38) using Sequencher v5.4.6 (Gene Codes, Ann Arbor, MI, USA).

Results

Sample characteristics and DNAm analysis quality

The investigated hairs were plucked from deceased individuals and were from different phases of the hair cycle, i.e. growing phase (anagen), transition phase (catagen) and resting phase (telogen) as given at the time of the individual's death. The amount of ssDNA after bisulfite conversion available for DNAm analysis was between 0.56 ng/ μ L and 30.6 ng/ μ L (mean = 6.04 ng/ μ L, median: 2.56 ng/ μ L, SD = 7.75 ng/ μ L). The DNAm of 18 samples could not be successfully determined as either the amount of PCR product and/or sequencing coverage by MPS was too low (15 cases) and the bisulfite conversion efficiency below 98% (3 cases). The obtained results revealed differences in PCR efficiency between the different analyzed loci. This issue has to be considered generally in hair samples as these often contain a low content of DNA and/or fragmented DNA. The *F5* and *TRIM59* loci were successfully analyzed in all of the 31 samples evaluable but *HOXC4* was successfully analyzed in only 14 samples.

Dependency of DNA methylation on age

The Spearman correlation coefficient (ρ) and the corresponding 95% CI were used to evaluate the correlation between the DNAm for each CpG site and the age of the individual. The 95% CI calculation for *MEIS1-AS3* and *HOXC4* resulted in a large range of more than ± 0.4 with respect to the obtained coefficient (*MEIS1-AS3* 0.17, CI –0.34, +0.6, *HOXC4_2* –0.33, CI –0.73, 0.24), which is also caused by the lower number of samples successfully analyzed for these markers. Therefore, the obtained correlation values of these two markers were not considered as interpretable and the markers not further considered in the study. The CpG site with the highest (ab-

solute) p value for each of the other eight loci is provided together with the 95% CI in **Table 1** and visualized in **Fig. 1**. A high correlation between the DNAm and age (absolute p value between 0.6 and 1) was observed for *ELOVL2*, *KLF14*, *RPA2*, *TRIM59*, and *ZYG11A*. A moderate correlation (absolute p value between 0.4 and 0.6) was determined for *NKIRAS2*. No age dependency was found for the analyzed CpG sites in *F5*, and *DDO*. The Spearman's ρ values of different CpG sites within the same amplicon were often very similar and can be affected by single outliers due to sample size. The calculated 95% CI of the highly correlated markers supports that these markers indeed show an age-dependency (at least moderate) even considering the sample number.

Investigation of interindividual variation

The correlation between age and age-dependent DNAm is not perfect. Influences on the epigenome during life, the genomic background and technical factors can affect the observed DNAm variability [7, 8, 20]. Looking at the genomic background (using unconverted DNA) of the eight DNAm loci considered for the age correlation analysis, no SNPs were found in the regions analyzed at the loci *DDO*, *ELOVL2*, *NKIRAS2*, and *TRIM59*. The SNPs without a direct effect on the CpG positions analyzed were detected for *F5* (9x rs2269648, 3x rs146501594) and *RPA2* (1x rs149574926). Furthermore, frequently occurring SNPs in *ZYG11A* (27x rs533935, 27x rs534070) were also found in a heterozygous or homozygous state in the individuals of this study. Two additional SNPs leading to removal of a CpG site were detected at CpG positions of loci *KLF14* (1x rs542947804) and *ZYG11A* (1x rs564173977). As the CpG position of one chromosome (heterozygote) was affected the measured DNAm value is based only on the CpG of the other chromosome.

An impact on the measured DNAm due to technical/stochastic variation has to be considered in hair, given the high variation in quality and quantity of the DNA available for analysis. A low ssDNA

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J. Naue · J. Winkelmann · U. Schmidt · S. Lutz-Bonengel

Analysis of age-dependent DNA methylation changes in plucked hair samples using massive parallel sequencing

Abstract

The analysis of age-dependent DNA methylation changes is a valuable tool in epigenetic research and forensic genetics. With some exceptions, most studies in the past concentrated on the analysis of blood, buccal, and saliva samples. Another important sample type in forensic investigations is hair, where age-dependent DNA methylation has not been investigated so far. In this pilot study a deeper look was taken at the possibilities and challenges of DNA methylation analysis in hair. The DNA methylation of selected age-dependent 5'-C-phosphate-G-3' (CpG) sites were characterized for their potential use as a biomarker for age prediction using plucked hair samples and massive parallel sequencing. Plucked hair roots of 49 individuals were included in the study. The DNA methylation of 31 hairs was successfully

analyzed. The DNA methylation pattern of 10 loci, including *ELOVL2*, *F5*, *KLF14*, and *TRIM59*, was determined by amplicon-based massive parallel sequencing. Age-dependent changes were found for several markers. The results demonstrate the possible use of already established age-dependent markers but at the same time they have tissue/cell type-specific characteristics. Special challenges such as low amounts of DNA and degraded DNA as well as the possible heterogeneous cellular composition of plucked hair samples, have to be considered.

Keywords

DNA methylation markers · Forensic epigenetics · Next Generation Sequencing · Aging · Amplicon based bisulfite sequencing

Analyse von altersabhängigen Veränderungen der DNA-Methylierung in ausgezupften Haarproben mittels massiver paralleler Sequenzierung

Zusammenfassung

Die Analyse von altersabhängigen Veränderungen der DNA-Methylierung ist ein wertvolles Werkzeug in der epigenetischen Forschung und forensischen Genetik. Mit einigen Ausnahmen konzentrierten sich die meisten Studien in der Vergangenheit auf die Analyse von Blut-, Wangen- und Speichelproben. Ein weiterer wichtiger Probentyp in forensischen Untersuchungen sind Haare, deren altersabhängige DNA-Methylierung bisher nicht untersucht wurde. In dieser Pilotstudie wurde ein tieferer Blick auf die Möglichkeiten und Herausforderungen der DNA-Methylierungsanalyse in Haaren geworfen. Die DNA-Methylierung ausgewählter altersabhängiger CpG-Stellen wurde auf ihre potenzielle Verwendung als Biomarker für die Altersvorhersage mittels gezupfter Haarproben und massiver Parallelsequenzierung charakterisiert. Gezupfte Haarwurzeln von 49 Individuen wurden in die Studie einbezogen. Die DNA-Methylierung

von 31 Haaren wurde erfolgreich analysiert. Das DNA-Methylierungsmuster von 10 Loci, darunter u. a. *ELOVL2*, *F5*, *KLF14* und *TRIM59*, wurde mit Hilfe von Amplicon-basierter massiver paralleler Sequenzierung bestimmt. Für mehrere Marker wurden altersabhängige Veränderungen gefunden. Die Ergebnisse zeigen die mögliche Verwendung bereits etablierter altersabhängiger Marker, die aber gleichzeitig gewebe-/zelltypspezifische Eigenschaften aufweisen. Besondere Herausforderungen wie geringe DNA-Mengen und degradierte DNA sowie die mögliche heterogene zelluläre Zusammensetzung von Haarproben müssen berücksichtigt werden.

Schlüsselwörter

Quantifizierung · DNA-Methylierungsmarker · Forensische Epigenetik · Next Generation Sequencing · Altern · Amplicon-basierte Bisulfittsequenzierung

Table 1 Spearman correlation between age and DNAm. The Spearman correlation (ρ) value of the 5'-cytosine-phosphate-guanine-3' (CpG) site with the highest correlation for each locus is presented together with the 95% confidence interval (CI). In this study, a high correlation (above 0.6) between DNAm and age was measured for *ELOVL2*, *KLF14*, *RPA2*, *TRIM59*, and *ZYG11A*. No age-dependency was seen in case of *DDO*, and *F5*. The p -value was adjusted to multiple testing

Marker	CpG (n)	GRCh38	ρ	95% CI	Adjusted p -value	Samples
DDO	1	chr6: 110415569	-0.17	[-0.57, 0.3]	0.51911	20
ELOVL2	1	chr6: 11044628	0.88	[0.76, 0.94]	<0.00001	29
F5	1	chr1: 169586775	0.18	[-0.19, 0.5]	0.38404	31
KLF14	2	chr7: 130734358	0.81	[0.57, 0.92]	0.00008	20
NKIRAS2	2	chr17: 42025398	-0.47	[-0.72, -0.13]	0.01491	29
RPA2	1	chr1: 27915022	0.70	[0.42, 0.86]	0.00030	25
TRIM59	6	chr3: 160450192	0.90	[0.79, 0.95]	<0.00001	31
ZYG11A	4	chr1: 52843096	0.78	[0.56, 0.89]	0.00001	27

GRCh38 genetic reference, *chr* chromosome

amount after bisulfite conversion in some samples did not explain all of the unexpected results, as samples with high DNA input were also affected. To additionally exclude that outliers might show a higher divergence due to a slightly lower coverage in MPS, a closer look was taken at loci of samples included in the final analysis that showed a coverage in between the minimum threshold and the anticipated number of reads (600–800 reads); however, a systemic bias due to the lower coverage was not identified as loci with a lower coverage did not show a higher tendency to be an outlier. Generally, most variation in the DNAm was observed in *DDO* and *F5*. Therefore, it can be assumed that *DDO* and *F5* do indeed show a higher deviation in hair samples due to biological and not technical reasons. This variation seen in hair is in accordance with investigations of other tissues for *DDO* but stands in contrast to *F5*, which showed relatively stable results in other tissues (see below and [23]).

Comparison with the DNAm of other tissues

The DNAm results from other tissues of the deceased individuals included in this study were either available [23] or newly generated. In total tissues for 25 of the 31 individuals with successfully analyzed hair samples were available for comparison (cf. “Material and methods”). The DNAm values of loci which were found to have a strong (*ELOVL2*, *KLF14*, *RPA2*, *TRIM59*, *ZYG11A*) and moderate

(*NKIRAS2*) correlation with age in hair, were also identified as good candidates for age prediction in blood, buccal swabs, skeletal muscle, brain and bone in the previous study [23]. This result is also supported by the analysis of the additional tissue samples; however, it has to be considered that the correlation is not always the best at the same CpG site. No age dependency was found for *F5* in hair as for muscle. The *DDO* locus showed no age dependency, which is a discrepant result with respect to other tissues where a moderate or strong correlation with age was found. The sample size in the study and the high interindividual DNAm variability observed in hair might mask an underlying significant but small age-dependent change.

For detailed comparison between 3 forensically relevant tissues, 23 individuals with DNAm values available from blood, buccal swabs and hair were considered. The DNAm results of the six age-dependent markers showed a generally lower DNAm for *ZYG11A* in hair (■ Fig. 2). In *ELOVL2*, *KLF14*, *TRIM59*, the DNAm in hair was close to the DNAm of blood, and for *NKIRAS2* values were between those of blood and buccal cells. When an individual showed a DNAm value lying outside the range of values expected in a given tissue, this individual never showed outlying DNAm values in all of the tissues. Interestingly, in the case of a 55-year-old individual, a far higher DNAm than expected was measured at *KLF14_2* in blood and buccal cells but not in hair. At least 5 ng of

DNA were analyzed in all three sample types from this individual but technical reasons cannot be completely excluded.

The CpG site within a locus with the strongest age correlation in hair analyzed within this study differed from the previously identified CpG sites in blood with the exception of *NKIRAS2* (CpG 2). In contrast, the most useful CpG sites of *TRIM59* (CpG 6), *ZYG11A* (CpG 4), *RPA2* (CpG 1), and *NKIRAS2* (CpG 2) are shared between buccal swabs and hair, which could be due to the shared cell type of keratinocytes; however, closely located CpG sites often had a nearly identical strength of correlation (cf [21, 23]), which indicates that multiple CpG sites per locus correlate with age in all three tissues and are comparable. Nevertheless, the change of %DNAm per year, as indicated by the slope in the case of a linear correlation, varied for the markers. For example, a higher accumulation of DNAm with age at *RPA2_1* was seen in the buccal swab samples compared to blood, and hair (■ Fig. 2). Additionally, the starting DNAm (in children) differed between tissues, and a 10% difference of the DNAm, e.g. in *ELOVL2_1*, has to be considered, even if the change per year is the same afterwards in buccal swab and hair samples (■ Fig. 2). These general observations were made in all markers and tissues analyzed albeit of different intensity.

Discussion

Possible effects on DNAm results due sample type and study cases

In this study, plucked head hair from deceased individuals was used for DNAm analysis. To avoid possible effects of a long agonal period or putrefaction, only individuals suddenly deceased (e.g. by traffic accident) and without signs of putrefaction were analyzed in this study. An increased time of agony might affect the DNAm and putrefaction leads to DNA degradation [1]. The collected hairs can originate from different phases of the hair cycle, as human hair growth is not synchronized, which may result in varying DNAm patterns [16, 24]. Additionally, it has to be considered that the number of

Fig. 1 ▲ DNAm of 10 CpG sites from age-dependent loci. The DNAm values for one selected CpG site of every locus depending on the individual's age are shown (X axis in years). The 5'-cytosine-phosphate-guanine-3' (CpG) site presented had the highest Spearman value within this study. For better visualization, the Y axis was adjusted to the range of observed DNAm values and a linear regression line included (shaded area shows the 95% confidence interval of the regression line)

cells obtained by plucking varies and that multiple cell types, such as keratinocytes, melanocytes, mesenchymal cells, as well as stem cells can be (involuntarily) included in the analysis of plucked hairs [27]. In this study, DNA extraction from the plucked hair led to a wide range in the quantity and—as shown by the PCR results—probably also in the quality of DNA. A direct correlation between the extracted DNA amount and the (roughly graded) number of attached cells was not seen.

Additionally, as samples were obtained during routine autopsies limited information on biological characteristics, such as disease, lifestyle and environmental influences was available, which cannot be excluded as possible sources for DNAm outliers. For example, factors such as hair graying due to the loss of active melanocytes of single hairs could be of interest as not all hairs are affected at the same time point and hair graying is generally associated with age [10]. Also, the study concentrated on head hair, and differences in the DNAm between head and body hair cannot be excluded as these are present as different types of hair [18]. Analysis of larger cohorts could

enable investigation of a possible effect of changes in hair color and hair location on age-dependent DNAm levels. The SNP analysis of the regions of interest was performed to investigate if specific outliers were caused by a variance of the DNA sequence itself. An SNP was found at a CpG site itself only in rare cases. This leads to the removal of a CpG site and can have a direct impact on the overall DNAm. The observed heterozygous mutations at the CpG sites KLF14_1, and ZYG11A_14 did not result in an outlier detection. It can be discussed if the non-affected homologous chromosome outbalances the loss in detectable DNAm. A possible impact of SNPs on a CpG site in close proximity could not be determined in this study; further evaluation would require more samples containing the SNPs under question.

Technical considerations

Hairs are a challenging sample source, which makes it difficult to differentiate between technical and biological variation of the true DNAm. Technical variation can either be directly caused by an inefficient analysis or due to stochastic

effects in the case of low DNA amounts or quality of the analyzed hair. Technical difficulties of the assay may result from suboptimal primer binding and unbalanced amplicon pooling. Assay optimization (e.g. change of the DNA polymerase) might improve PCR efficiency resulting in more suitable analysis of all markers in the case of low quality DNA. Especially, the PCR efficiency of *HOXC4* and *MEIS1-AS3* was lower resulting in less successfully analyzed samples. The calculated 95% CI of the obtained ρ of the study samples showed a wide possible range and the determined correlation coefficients were therefore not considered as reliable to demonstrate the exact correlation with age. Furthermore, ssDNA quantity was fluorometrically determined, not reflecting the DNA quality of the sample which is additionally fragmented during the bisulfite conversion step [19]. Therefore, the determined ssDNA amount in this study has been considered as an estimation of the ssDNA amount used for PCR. Strong degradation might be an explanation for outliers with initially high ssDNA input of DNA. In the case of fragmented DNA, MPS results (even with a high cover-

Fig. 2 ▲ DNAm for cases where DNAm for all three tissues (hair, blood, and buccal swab) was obtained. In 23 cases, blood, buccal swabs, and hair samples were successfully analyzed from the same individuals. For each marker, only cases with DNAm values for all three tissues were considered for better comparison of the correlation with age and the range of the DNAm level with aging. The DNAm values for one selected 5'-cytosine-phosphate-guanine-3' (CpG) site of every locus in dependence of the individual's age are shown (X axis in years). The CpG site presented showed the highest Spearman correlation coefficient for hair in this study. For a better visualization, the Y axis was adjusted to the range of observed DNAm values, and a linear regression line included

age) might result from the amplification of only a few template molecules during the initial PCR. These results would then not reflect the actual DNAm of the initial cell material but rather a selection of a few molecules of high quality. Even if short nuclear DNA fragments can be detected in a balanced analysis and therefore reflect the true DNAm value of the sample, the DNAm value of a few molecules will result in high stochastic effects between samples. Another aspect is that external DNA present on the surface of a hair would have an impact on the measured DNAm. Deep cleaning of the hair, e.g. by using NaClO, was not possible as this procedure would have destroyed a lot of material or the hair follicle itself. Therefore, it has to be considered that small traces of environmental DNA might have been present. Head hairs were collected during autopsy wearing gloves and handled carefully afterwards minimizing any external contamination. Only in the case of one sample, an initial mild lysis could not be avoided due to adhering blood; however, it cannot be completely excluded that attached cells (e.g. outer sheath cells) get damaged in case of a mild lysis.

Comparison to DNAm from other tissues

Multiple studies analyzing age-dependent DNAm from different tissues were performed in the past (e.g. [3, 5, 13–15, 21, 23]); however, a direct comparison is complicated due to differences in the studied individuals, sample collection, and the methods used for analysis and data processing. A comparison of age-dependent DNAm changes in a high number of various tissues was presented by Horvath in 2013 to develop the multi-tissue clock [13] but the samples included were obtained from various studies and sample populations. Therefore, a direct comparison between the DNAm and age from different tissues is limited, as individuals and the investigated age range differ between the studies. In our study, however, the results of the hair analysis could be compared to data from other tissues from the same individuals allowing a direct comparison between the tissue-dependent DNAm results. Differences between hair and other tissues were observed and only some of the DNAm markers also changed with age in hair, as seen before for the other

tissues [23]. Some DNAm markers show a strong correlation with age in hair as well as the other tissues; however, the mere comparison between the obtained correlation coefficients does not permit the conclusion that the age dependency and/or the kind of change of a given DNAm marker is universal. Strong correlations were obtained for several tissues and markers (cf. ■ Figs. 1 and 2) but tissue-specific DNAm values were still observed. Both the initial DNAm at birth as well as the speed of change with age differed between the tissues. This can be due to biological reasons, stochastic effects in DNAm changes as well as to the heterogeneity of cell types in tissues. The CpG site with the highest Spearman correlation coefficient (ρ) detected often differed between tissues; however, the values between neighboring CpG sites were often close and the obtained correlation coefficients and the statistical significance for each CpG site have to be further supported by larger sample sets.

Conclusion and outlook

The current article presents a pilot study on the general feasibility of DNAm anal-

ysis from hair. Determination of the DNAm of selected DNA regions from plucked head hairs was possible. For application in forensics, especially robust age-dependent markers with a strong correlation between DNAm and age should be further considered. The most promising markers for a final assay to analyze the DNAm for age prediction based on hair samples were *ELOVL2*, *KLF14*, *RPA2*, *TRIM59*, and *ZYG11A*. These markers can then be incorporated into hair-specific mathematical models for age prediction. The analysis in this study was performed in a singleplex approach but future multiplexing will provide the possibility to include more DNA in one analysis as splitting of the sample can then be omitted. The analysis of different hair phases and a diversely composed population of cells might have led to higher variation or superimposed specific age-dependent changes of loci in specific cell types and phases of the hair cycle. Therefore, the analysis of single hairs of an individual as well as of specified hair cycle phases could help to shed light onto the variability or robustness of age-dependent DNAm markers due to different phases of cell cycles and cell types. This would provide more information about the usability for hair found at a crime scene. Furthermore, research is needed to investigate if enough material with high quality of DNA for a DNAm analysis can still be obtained from shed hair in contrast to plucked hair, and if a comparable interpretation is possible with respect to the variability of cell type composition observed between hairs. A general limitation is that no reliable DNAm analysis is possible if only a hair shaft is available, even if a few short nuclear DNA fragments might be present.

Corresponding address

Jana Naue

Institute of Forensic Medicine, Medical Center—University of Freiburg, Faculty of Medicine, University of Freiburg
Freiburg, Germany
jana.naue@uniklinik-freiburg.de

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Declarations

Conflict of interest. J. Naue, J. Winkelmann, U. Schmidt and S. Lutz-Bonengel declare that they have no competing interests.

Ethical standards. Research involving human participants. Approval for tissue sampling and DNA methylation analysis was given by the ethics committee of the University of Freiburg, Germany (263/10, amendment 397/16). All procedures performed in studies involving human participants were in accordance with the ethical standards of the ethics committee of Faculty of Medicine, University of Freiburg, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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E. Doberentz · J. Ulbricht · B. Madea

Institut für Rechtsmedizin, Universitätsklinikum Bonn, Bonn, Deutschland

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Im o. g. Beitrag wurde versehentlich bei Abb. 1 eine falsche Quelle angegeben. Die korrekte Quelle lautet: (Nach Schmidt [12]). Das Literaturverzeichnis wurde unnummeriert.

Der Originalbeitrag wurde korrigiert. Für diesen Fehler möchten wir uns entschuldigen.

Korrespondenzadresse

PD Dr.med. E. Doberentz

Institut für Rechtsmedizin, Universitätsklinikum Bonn

Stiftsplatz 12, 53111 Bonn, Deutschland

edoberentz@uni-bonn.de

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