

# Potential forensic application of DNA methylation profiling to body fluid identification

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**Abstract** DNA analysis of various body fluid stains at crime scenes facilitates the identification of individuals but does not currently determine the type and origin of the biological material. Recent advances in whole genome epigenetic analysis indicate that chromosome pieces called tDMRs (tissue-specific differentially methylated regions) show different DNA methylation profiles according to the type of cell or tissue. We examined the potential of tissue-specific differential DNA methylation for body fluid identification. Five tDMRs for the genes DACT1, USP49, HOXA4, PFN3, and PRMT2 were selected, and DNA methylation profiles for these tDMRs were produced by bisulfite sequencing using pooled DNA from blood, saliva, semen, menstrual blood, and vaginal fluid. The tDMRs for DACT1 and USP49 showed semen-specific hypomethylation, and the tDMRs for HOXA4, PFN3, and PRMT2 displayed varying degrees of methylation according to the type of body fluid. Preliminary tests using methylation-specific PCR for the DACT1 and USP49 tDMRs showed that these two markers could be used successfully to identify semen samples including sperm cells. Body fluid-specific

differential DNA methylation may be a promising indicator for body fluid identification. Because DNA methylation profiling uses the same biological source of DNA for individual identification profiling, the determination of more body fluid-specific tDMRs and the development of convenient tDMR analysis methods will facilitate the broad implementation of body fluid identification in forensic casework.

**Keywords** Body fluid identification · DNA methylation · Tissue-specific differentially methylated region

## Introduction

Body fluids found at a crime scene provide crucial information to link evidence and the crime. DNA obtained from body fluids such as blood, saliva, and semen can be used to identify the donor of the biological material, and the determination of the type and origin of the biological material can help reconstruct crime scenes. However, conventional body fluid identification using serological or immunological tests cannot positively confirm the presence of certain biological fluids. Even when a forensic stain test is positive for a particular body fluid, the available DNA profile might come from a different body fluid or tissue source [1]. Therefore, a confirmatory method that allows the identification of the cellular source of DNA profiles would be a valuable tool in reconstructing crime scenes.

Recent advances in forensic genetics have revealed that RNA extracted from the same biological source as the DNA profile can be used for body fluid identification by quantifying RNA that is specific for certain body fluids [2–11]. Contrary to popular belief, some RNA markers were successfully amplifiable in aged samples such as 16-year-old bloodstains [11]. There is a concern, however, that

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forensic scientists unfamiliar with handling RNA will bypass such a body fluid identification method and proceed straight to the DNA analysis of forensic samples. Since DNA is easier to handle than RNA and is used for individual identification, a body fluid identification method using DNA itself would be beneficial for forensic casework.

DNA methylation, which occurs at the 5'-position of the pyrimidine ring of cytosines in CpG dinucleotides, is a genetically programmed DNA modification in mammals [12, 13] that plays an important role in mammalian development and cellular differentiation, and allows cells to maintain different characteristics by controlling and modulating gene expression through chromatin structure [14–16]. DNA methylation patterns are susceptible to change in response to environmental stimuli such as diet or stress, and are most vulnerable to a change during early in utero development. Once DNA methylation patterns are established through cellular differentiation, however, they display a limited dynamic range in normal conditions, giving various cells and tissues a unique cell- or tissue-specific DNA methylation profile [17–21]. Genome-wide analysis of DNA methylation also indicates that numerous tissue-specific differentially methylated regions (tDMRs) exist in the mammalian genome [22–24].

We selected candidate tDMRs expected to show differential DNA methylation profiles in various body fluids, and produced DNA methylation profiles for these tDMRs using pooled DNA samples from blood, saliva, semen, menstrual blood, and vaginal fluid. We discuss the potential forensic application of DNA methylation profiles of these tDMRs for body fluid identification.

## Materials and methods

### Samples

Body fluid samples (venous blood, saliva, semen, menstrual blood, and vaginal fluid) were collected from volunteers using procedures approved by the Institutional Review Board of Severance Hospital, Yonsei University in Seoul, Korea. Sixteen participants, 10 males and 6 females, gave their informed consent in writing after the aims and procedures of the study were explained. Blood was collected by lancet and 5  $\mu$ L aliquots were stored frozen with anticoagulant. Saliva was collected in a microcentrifuge tube and 100  $\mu$ L aliquots were stored frozen. Freshly ejaculated semen was collected in plastic cups and stored frozen until it was dried onto sterile cotton swabs. Menstrual blood and vaginal fluids were collected using sterile cotton swabs and allowed to dry at room temperature. Dried swabs were stored frozen until needed. DNA was extracted from an aliquot or a single cotton swab using a QIAamp DNA Investigator Kit (Qiagen, Hilden,

Germany) according to the manufacturer's instructions. Extracted DNA was quantified using a NanoDrop<sup>®</sup> ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

### Selection of candidate markers for body fluid identification

Five tDMRs were selected to generate DNA methylation profiles using body fluid samples (Table 1). Two previously reported testis-specific DMRs were selected as candidate semen-specific markers [19] because the testes produce and store the millions of sperm cells that make up the main source of semen DNA. Three tDMRs that show different methylation profiles in blood, brain, muscle, and spleen tissue were selected as candidate blood-specific markers based on the results of a previous report [23]. Genomic sequences and CpG islands were obtained from the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>). PCR primers for bisulfite-treated DNA analysis were designed using the Methprimer program (<http://www.urogene.org/methprimer/index1.html>) [25] or from sequences used in a previous report [19] (Table 2).

### Bisulfite treatment and sequencing

DNA samples of the same body fluids across individuals were pooled together such that 100 ng of DNA from each participant was included. The pooled DNA was modified by bisulfite treatment following the conversion protocol of the Imprint<sup>®</sup> DNA Modification Kit (Sigma-Aldrich Inc., St. Louis, MO, USA). Bisulfite-treated DNA was amplified in a 25- $\mu$ L reaction volume containing 1  $\mu$ L template DNA, 1.5 U AmpliTaq Gold<sup>®</sup> DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 2.5  $\mu$ L Gold ST<sup>\*</sup>R 10 $\times$  Buffer (Promega, Madison, WI, USA), and 0.4  $\mu$ M of each primer. PCR cycling was conducted in a PTC-200 DNA engine (MJ Research, Waltham, MA, USA) under the following conditions: 95 $^{\circ}$ C for 11 min; 37–39 cycles of 94 $^{\circ}$ C for 45 s, 55 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 45 s; and a final extension at 72 $^{\circ}$ C for 7 min. PCR products were cloned into a PCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector using the TOPO TA Cloning<sup>®</sup> Kit (Invitrogen, Carlsbad, CA, USA). Thirty and 20 positive clones were isolated from each male and female body fluid, respectively. Clones were sequenced using the M13 reverse primer and BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing results were analyzed in an ABI 3730xl DNA Analyzer (Applied Biosystems).

### DNA methylation profiling

To determine the methylation status of candidate markers in the various body fluid samples, sequencing data were aligned against in silico-converted genomic reference

**Table 1** Genomic information for candidate tDMRs for body fluid identification

Tissue	UCSC location (Mar. 2006)	CpG island	Gene	Function	References
Testis	chr14:58182690–58182995	Close to cpgi50	DACT1	Dapper 1 isoform 2	[19]
Testis	chr6:41881884–41882111	cpgi46	USP49	Ubiquitin carboxyl-terminal hydrolase 49	[19]
Blood	chr7:27135995–27136879	cpgi87	HOXA4	Homeobox protein Hox-A4	[23]
Blood	chr5:176758438–176760564	cpgi82	PFN3	Profilin-3	[23]
Blood	chr21:46905647–46905874	cpgi55	PRMT2	Protein arginine N-methyltransferase 2	[23]

sequences using BiQ Analyzer (<http://biq-analyzer.bioinf.mpi-sb.mpg.de/>) [26]. The output files were used for the BDPC web application (<http://biochem.jacobs-university.de/BDPC/>) to compile the derived information and compare results from each marker in the different body fluid samples [27]. The body fluids in the methylation map were also sorted using the BDPC web application with respect to the UPGMA clustering, which is based on the respective Pearson's correlation coefficient of the body fluids analyzed when compared with each other.

#### Statistical analysis

To evaluate the methylation profile of candidate markers to see whether they could be used to distinguish individual body fluids and identify the origin of specific body fluids effectively, statistical analyses were carried out using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Pairwise comparisons of methylation were made for each marker and CpG locus using chi-square test or Fisher's exact test as appropriate. Differences were statistically significant when *p* values were less than 0.01.

#### Preliminary test for semen identification using tDMRs for DACT1 and USP49

Methylation-specific PCR (MSP) was designed to differentiate methylated from unmethylated cytosines present in the DACT1 and USP49 tDMRs (Table S1). PCR primers were designed using the Methprimer program or Primer 3

program (<http://frodo.wi.mit.edu/primer3/>) [28]. To facilitate the analysis of degraded DNA, the sizes of the MSP amplicons used were under 150 bp. One to 50 ng of DNA obtained from the body fluids of two males and females were subjected to bisulfite-conversion using the Imprint<sup>®</sup> DNA Modification Kit. Bisulfite-treated DNA was amplified in a 25- $\mu$ L reaction volume containing 1  $\mu$ L template DNA, 1.5 U AmpliTaq Gold<sup>®</sup> DNA Polymerase, 2.5  $\mu$ L Gold ST<sup>®</sup>R 10 $\times$  Buffer, and 0.4  $\mu$ M of each primer. PCR cycling was conducted in a PTC-200 DNA engine under the following conditions: 95°C for 11 min; 35–36 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. Results were obtained immediately following PCR amplification and gel electrophoresis without further sequencing analysis. EpiTect PCR Control DNA (Qiagen) containing both bisulfite converted methylated and unmethylated DNA was used for control PCRs in all reactions. Semen samples of vasectomized men were obtained from three volunteers and were examined using procedures as previously described. To test the in vitro stability of methylation markers, DNA was extracted from dried specimens (blood and saliva spotted onto a clean cotton tissue and semen, menstrual blood, and vaginal fluid absorbed with cotton swabs) that had been stored at an ambient temperature in the shade for 30 days and amplified using MSP. DNA extracted from paraffin-embedded tissues was also subjected to MSP and bisulfite sequencing to test whether an examination of differential DNA methylation using the two methods can be applied to the fragmented DNA.

**Table 2** Primers used for bisulfite sequencing

Gene	UCSC location (Mar. 2006)	Forward primer	Reverse primer	No. CpG	Size (bp)
DACT1	chr14:58182690–58182995	GGAAGAGAGG <u>TTT</u> GTTTGGATTTTA	CTCTTCTCCCCTT <u>AAAAAA</u> ACTTT	14	306
USP49	chr6:41881884–41882111	GTTGAGTATTTGGAGGATGGAGTTTAT	CT <u>AAAAAA</u> ACTA <u>ACCAAC</u> ACCCCTC	22	228
HOXA4	chr7:27135937–27136325	TATTGGGGT <u>TGAAGAAA</u> AGTTTAA	CTAA <u>ACCT</u> AAAA <u>CAAAAA</u> ACC	19	389
PFN3	chr5:176759649–176760021	TTTGTA <u>AAGATT</u> AGTTTTTTTATTATAAT	<u>AACAAAC</u> CACACCTT <u>CCTACA</u> AAC	42	373
PRMT2	chr21:46905841–46906149	GGAAAGG <u>TTT</u> AGTTTGGAGT <u>TTTT</u> TA	CCCTC <u>AAAA</u> TCT <u>AAATCAA</u> CAAATA	21	309

PCR primers are specific for one strand of the bisulfite-converted DNA. During bisulfite conversion, unmethylated cytosines are chemically converted to uracils, which will be amplified as thymidine. The nucleotide T (A in complement sequence) that occurred upon the bisulfite conversion of unmethylated cytosine is underlined

## Results and discussion

### tDMRs for the genes DACT1 and USP49

All five candidate markers (tDMRs within exons of the genes DACT1, HOXA4, PFN3, PRMT2, and USP49) showed differential methylation profiles in blood, saliva, semen, menstrual blood, and vaginal fluid (Fig. 1, Table S2).

Body fluid-specific differences were most evident in the DNA methylation of tDMRs for the genes DACT1 and USP49 from semen. Since the tDMRs for DACT1 and USP49 are known to show hypomethylation in testes while displaying hypermethylation in other tissues (i.e., kidney, liver, heart, spleen, and muscle) [19], we expected that both would demonstrate hypomethylation in semen and hypermethylation in other body fluids (Fig. 1). The tDMR for DACT1 was unmethylated in 93% of clones from semen but hypermethylated in all of the clones from blood, saliva, menstrual blood, and vaginal fluid (Fig. S1). The tDMR for USP49 was hypomethylated for 97% of clones from semen, while almost all of the clones from the other body fluids were hypermethylated (Fig. S2). Statistical analysis also showed that methylation data obtained from blood, saliva, menstrual blood, and vaginal fluid were not significantly different from each other ( $p > 0.01$ ) and that semen differed from all the other body fluids at every individual CpG locus as well as in the overall methylation of the tDMRs for DACT1 and USP49 ( $p < 0.001$ ) (Tables S2, S3 and S4). Even though blood samples displayed a difference in overall methylation of the USP49 tDMR between males and females ( $p < 0.001$ ), the methylation at each CpG locus was not significantly different ( $p > 0.01$ ) (Table S4). Accordingly, the two testis-specific DMRs with semen-specific hypomethylation profiles appear to have potential applicability for the identification of semen.

### tDMRs for the genes HOXA4, PFN3, and PRMT2

The tDMRs for the HOXA4, PFN3, and PRMT2 genes were initially selected as blood-specific markers because these genes are reportedly methylated in blood but not in brain, muscle, or spleen tissue [23]. These previous findings lacked confirmation by a gene-specific investigation, however, and we found that all three tDMRs were methylated in both blood and other body fluid samples to varying degrees (Fig. 1). We further explored whether the varying degree of methylation of the three candidate markers could be used to identify various body fluids including blood.

The tDMR for HOXA4 showed hypomethylation in almost all of the clones from semen and in half of the clones from male saliva, menstrual blood and vaginal fluid,

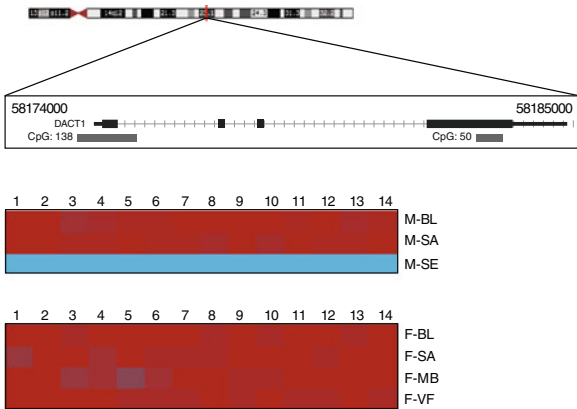
and displayed hypermethylation in all of the clones from blood and female saliva (Fig. S3). Although overall DNA methylation was statistically different in almost all body fluid pairs (Table S2), DNA methylation at each CpG locus was consistent with the observation shown in Fig. S3; blood and female saliva or menstrual blood and vaginal fluid had no distinguishing significant difference in DNA methylation except at one or two CpG loci ( $p > 0.01$ ), and the hypomethylation of semen differed from all the other body fluids except for vaginal fluid ( $p < 0.001$ ) (Table S5). The difference in the degree of methylation of male and female saliva was obvious ( $p < 0.001$ ), but could be confirmed by analysis of more clones (data not shown). Twenty one and 10 more clones analyzed had 52.3% and 90.7% of DNA methylated in male and female saliva, respectively, which demonstrates that the difference was not caused by analysis of an inappropriate number of sequenced clones. Consequently, the presence of an unmethylated clone of the tDMR for HOXA4 in some types of unmixed biological stains could be used to exclude the possibility of the presence of blood in a biological sample.

At the tDMR for PFN3, almost all analyzed body fluids displayed hypermethylation, with 80% or more CpG loci methylated (Fig. S4). In vaginal fluid, however, only about 65% of the CpG loci were methylated, and among all 42 CpG loci, four (CpG Nos. 1, 3, 16, and 18) were highly unmethylated. Statistical analysis also showed that the overall DNA methylation of vaginal fluid was significantly different from those of the other body fluids ( $p < 0.001$ ), and this difference was most evident at CpG Nos. 1, 3, 16, 18, 22, and 36 ( $p < 0.001$ ) (Tables S2 and S6). These results suggest the potential value of the several CpG loci in the PFN3 tDMR for the identification of vaginal fluid using site-specific DNA methylation analyses (e.g., Methylation SNaPshot, MethyLight, or others) [15].

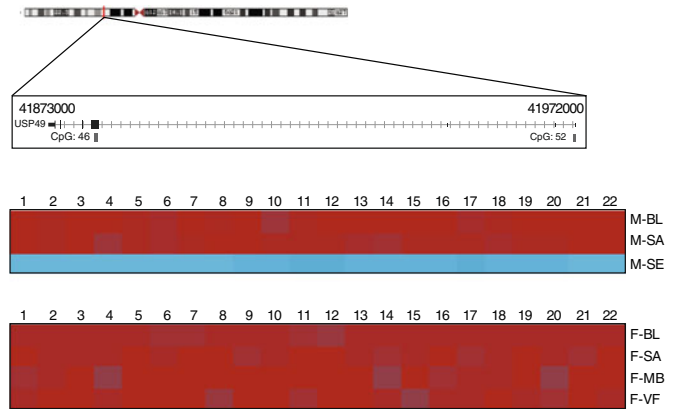
In contrast, the tDMR for PRMT2 showed hypomethylation in almost all of the clones from semen, but hypermethylation in more than half of the clones from menstrual blood and vaginal fluid. This suggests that the marker for PRMT2 may be useful not only to differentiate semen from all the other fluids tested based on hypomethylation, but also to differentiate menstrual blood and vaginal fluid from all the other body fluids based on hypermethylation (Fig. S5). However, contrary to this observation and the overall DNA methylation comparison, the DNA methylation at each CpG locus showed statistically significant differences only between semen and menstrual blood or between semen and vaginal fluid (Tables S2 and S7).

Collectively, these three tDMRs displayed similar hypo- or hypermethylation profiles in some different body fluids, but may still be useful when used in conjunction with DNA methylation profiling for semen-specific tDMRs. For example, the hypomethylation of the HOXA4 tDMR and

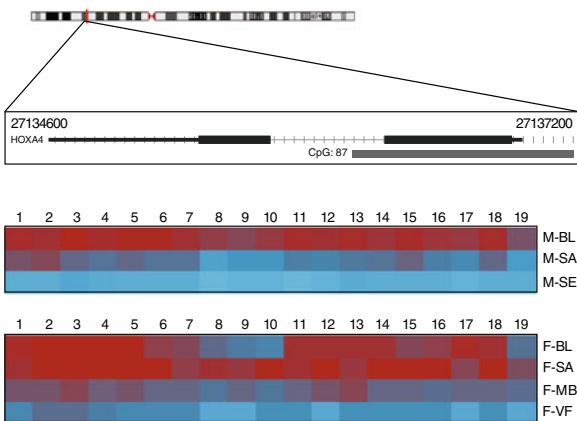
a. Chr14:58182690–58182995\_CGI50



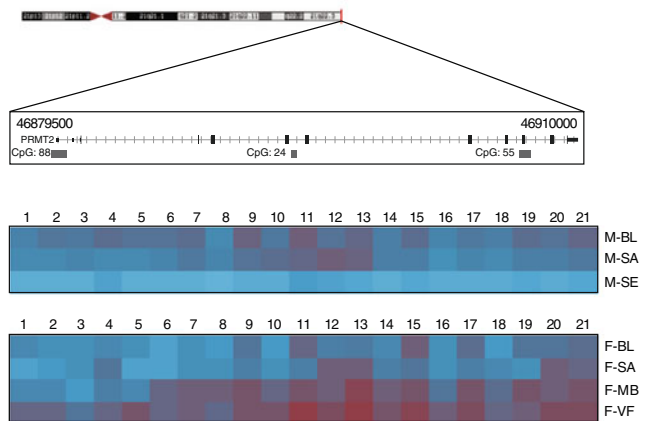
b. Chr6:41881884–41882111\_CGI46



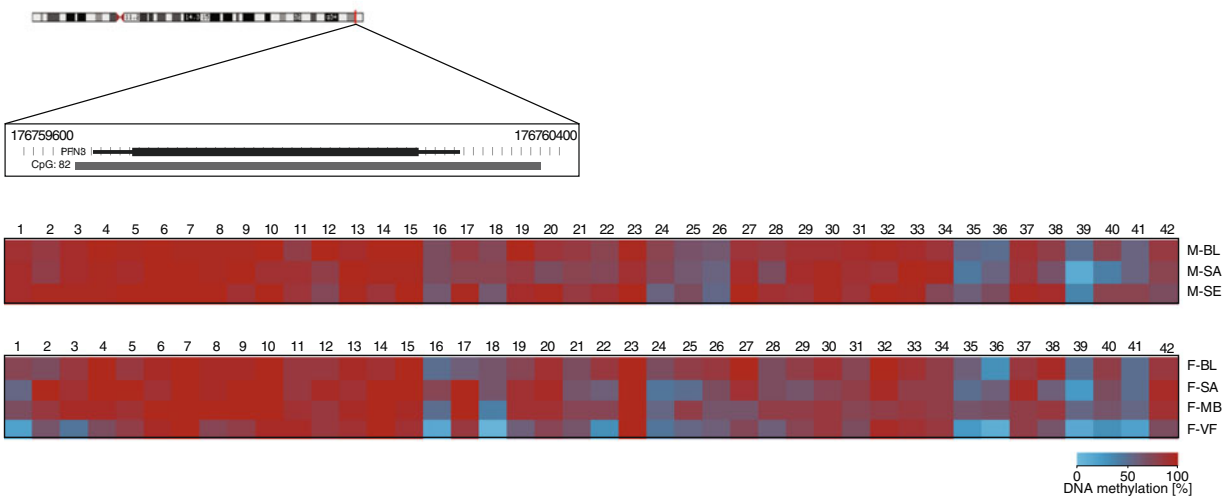
c. Chr7:27135937–27136325\_CGI 87



d. Chr21:46905841–46906149\_CGI 55



e. Chr5:176759649–176760021\_CGI 82



**Fig. 1** Differential DNA methylation of five tDMRs in various body fluids. The tDMRs for DACT1 (a), USP49 (b), HOXA4 (c), PRMT2 (d), and PFN3 (e) show evidence of body fluid-specific methylation by bisulfite sequencing. Chromosomal locations of the analyzed tDMR regions and adjacent CpG islands (CGIs) were indicated from the UCSC Genome Browser in Humans Mar. 2006 Assembly ([http://](http://genome.ucsc.edu)

[genome.ucsc.edu](http://genome.ucsc.edu)). The methylation heat map shows the average degree of methylation of all subcloned amplicons in each body fluid type. CpG positions are indicated by the number that corresponds to the order within each amplicon. *M-BL*, *M-SA*, *M-SE*, *F-BL*, *F-SA*, *F-MB*, and *F-VF* represent male blood, male saliva, semen, female blood, female saliva, menstrual blood, and vaginal fluid, respectively

hypermethylation of the PRMT2 and semen-specific DMRs may be useful for confirming the presence of menstrual blood or vaginal fluid. In addition, since the tDMR for PFN3 was highly unmethylated at several specific CpG loci in vaginal fluid, additional analyses of these loci could allow the identification of menstrual blood and vaginal fluid.

However, sex differences observed in the methylation profiles of the HOXA4 tDMR from male and female saliva made it difficult to apply that marker for body fluid identification. Considering that DNA methylation can control gene expression, the fact that the expression of HOXA4 was statistically significantly higher in oral squamous cell carcinoma samples than in normal oral mucosa samples implies that the DNA methylation of the HOXA4 tDMR might be susceptible to a change in response to environmental stimuli [29]. This would suggest that the HOXA4 tDMR would not be a good candidate marker for body fluid identification.

#### Screening and selection of markers using pooled DNA samples

A similarity plot and a dendrogram of the tissue clustering were obtained from pairwise comparisons of overall methylation of the tDMRs for DACT1, USP49, PRMT2, and PFN3 in blood, saliva, semen, menstrual blood, and vaginal fluid (Fig. S6). The negative value for semen in a similarity plot revealed that semen differs from all the other body fluids, and the dendrogram that displays hierarchical clustering of the body fluids tested shows that it is possible to differentiate semen and vaginal fluid from the other body fluid samples tested. Therefore, the combined use of tDMRs for DACT1, USP49, PRMT2, and PFN3 should facilitate the identification of semen and vaginal fluid.

Some aspects of the selection of markers and screening procedures used in the present study should be considered. The DNA methylation profiling in the present study was performed on pooled DNA from multiple individuals, but not all individuals have identical DNA methylation profiles. Since the methylation of the gene can repress gene expression [14, 15], the DNA methylation in half of the clones could indicate monoallelic expression of the gene, the presence of mixed cell populations with active and inactive gene expression, or differences among individuals in the pooled DNA samples. However, a recent study showed that DNA methylation patterns are more consistent between the same tissues from different people than between different tissues from the same individual [30], which suggests that the screening procedure using pooled DNA samples might be valid. Nevertheless, DNA quantification in the present study was performed using a NanoDrop® ND-1000 spectrophotometer rather than with real-time PCR. Accordingly, it is possible that co-purified

bacterial DNA and other materials that might absorb light at 260 nm were included in the pooled DNA samples, thereby introducing differences in the amount of individual input DNAs. To offset the potential for negative effects from unexpected ingredients and to minimize the stochastic effect for minor human genomic components during the subsequent PCR, a relatively large amount of DNA (100 ng of each input DNA from an individual) was used for pooled DNA methylation profiling in the present study. DNA methylation profiles would facilitate the screening of candidate markers by providing group average estimates, but this requires the further evaluation of selected markers from each individual. At minimum, the tDMRs or the CpG loci that showed hyper- or hypomethylation in almost all clones in a particular body fluid would probably have similar DNA methylation profiles in all individuals. Accordingly, the tDMRs for DACT1, USP49, and PFN3 will represent good candidate markers for the identification of semen or vaginal fluid. Moreover, the potential problems involving mixtures could also be addressed by using the tDMRs for the DACT1 and USP49 genes because they show nearly “all-or-none” DNA methylation profiles that depend upon the type of body fluid.

#### Identification of semen using tDMRs for DACT1 and USP49

To test whether an examination of differential DNA methylation can be applied to body fluid identification, we tentatively designed MSP to differentiate methylated from unmethylated cytosines present in the DACT1 and USP49 tDMRs (Table S1). During bisulfite conversion, unmethylated cytosines are chemically converted to uracils, and accordingly, specific amplification of methylated or unmethylated DNA can be accomplished by appropriate primer design. As expected, MSP for the DACT1 and USP49 tDMRs could be used to differentiate semen from blood, saliva, menstrual blood, and vaginal fluid samples because of the hypomethylation of DNA in semen and hypermethylation of DNA in other body fluids tested (Fig. S7). Semen samples obtained from vasectomized men were also analyzed and little DNA was obtained; however, it showed the same hypermethylation pattern as found in the other body fluids, which suggests that the DACT1 and USP49 tDMRs are sperm-specific markers (Fig. S8). Another testis-specific DMR, VASA, which showed hypomethylation in testis but hypermethylation in other tissues, actually exhibits germ cell-specific expression and DNA hypomethylation pattern [31].

A test using 30-day-old samples (blood and saliva spotted onto a clean cotton tissue and semen, menstrual blood, and vaginal fluids absorbed with cotton swabs) demonstrated that DNA methylation and unmethylation

were stable over time (Fig. S9). Because the MSP primers were designed to produce small amplicon products, degraded DNA was expected to be amplifiable using those primers. MSP using paraffin-embedded tissues confirmed this by showing good amplification results; however, bisulfite sequencing primers, which yield an amplicon size >300 bp, failed to allow the production of amplicons when the same samples were used (Fig. S10). In addition, 1 ng of DNA was shown to be sufficient to produce a DNA methylation profile using bisulfite sequencing primers, although almost 1 µg of DNA (100 ng of each individual DNA) was used for screening in the present study (Fig. S11). The Imprint® DNA Modification Kit is known to work well with a 50 pg DNA in DNA methylation analysis using MSP following bisulfite conversion (refer to MOD50 at <http://www.sigmaldrich.com/>). DNA methylation analysis using the bisulfite conversion procedure is sensitive and can be applied to aged and degraded samples; therefore, body fluid-specific DMRs can be used effectively for forensic body fluid identification.

Prospects for the application of differential DNA methylation analysis to body fluid identification in forensics

As numerous tDMRs exist in the mammalian genome [22–24], identification of additional body fluid-specific DMRs is expected to spur the development of promising body fluid identification methods for forensic applications. Actually, a recent study reported the use of 15 differentially methylated loci for tissue identification, which was achieved by screening 250 genomic loci using methylation-sensitive enzyme digestion followed by PCR analysis of pooled DNA samples of blood (venous/menstrual), saliva, semen, skin epidermis, urine, and vaginal secretion [32]. The selected loci enabled the detection of blood, saliva, semen, and skin epidermis by sequential procedures, comprising methylation-sensitive restriction enzyme digestion, multiplex amplification of specific genomic targets with fluorescent-labeled primers, capillary electrophoresis of amplicons, and signal analysis. Because the system is automatable and allows combining tissue identification with DNA profiling for individual identification, differential DNA methylation may hold great promise beyond its projected use in body fluid identification.

As the authors indicated [32], however, tissue identification using the methylation-sensitive restriction enzymes relies on efficient digestion of the template DNA, and the screening method used permitted the examination of only one CpG locus from each amplicon. Meanwhile, MSP in the present study specifically amplified correct targets using primers that anneal to bisulfite-converted DNA, and the DNA methylation profiles produced by bisulfite sequencing

provided high-resolution mapping of DNA methylation patterns by revealing the methylation status of every CpG loci in each amplicon. Because differential methylation of imprinted genes has long been studied to determine the parental origin of alleles in paternity cases [33, 34], many forensic experts are familiar with DNA methylation profiling and the associated techniques of methylation-sensitive enzyme digestion followed by PCR analysis, bisulfite sequencing, MSP, and methylation-sensitive single-nucleotide primer extension. Therefore, there should be no problems in developing more effective analytical methods and applying differential DNA methylation analysis to forensic body fluid identification in the near future. Moreover, since it is now possible to screen more than 450,000 methylation sites per sample at single-nucleotide resolution (refer to [http://www.illumina.com/products/methylation\\_450\\_beadchip\\_kits.ilmn](http://www.illumina.com/products/methylation_450_beadchip_kits.ilmn)), the identification of more informative tDMRs for body fluid identification should be forthcoming. However, an association between disease and changes in the DNA methylation status of candidate markers may need to be considered during the selection of markers for body fluid identification because a growing number of diseases have been found to be associated with aberrant DNA methylation patterns.

## Conclusion

The analysis of tissue-specific differential DNA methylation was proposed as a promising new method for the identification of body fluids. Five tDMRs were tested using bisulfite sequencing analysis of DNA obtained from pooled samples of blood, saliva, semen, menstrual blood, and vaginal fluid collected from multiple volunteers. The tDMRs for DACT1 and USP49 showed semen-specific hypomethylation profiles, and the tDMRs for HOXA4, PFN3, and PRMT2 displayed varying methylation profiles according to the type of body fluid. The combined use of tDMRs for DACT1, USP49, PRMT2, and PFN3 could be used for the identification of semen and vaginal fluid. Preliminary tests using methylation-specific PCR for the DACT1 and USP49 tDMRs showed that these two markers could be used to successfully identify semen samples including sperm cells. Future genome-wide DNA methylation analysis using various body fluid samples will be useful to identify additional body fluid-specific tDMRs and enable the subsequent development of efficient analysis methods for forensic casework.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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