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Genome-wide methylation profiling and a multiplex construction for the identification of body fluids using epigenetic markers

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ABSTRACT

The identification of body fluids found at crime scenes can contribute to solving crimes by providing important insights into crime scene reconstruction. In the present study, body fluid-specific epigenetic marker candidates were identified from genome-wide DNA methylation profiling of 42 body fluid samples including blood, saliva, semen, vaginal fluid and menstrual blood using the Illumina Infinium HumanMethylation450 BeadChip array. A total of 64 CpG sites were selected as body fluid-specific marker candidates by having more than 20% discrepancy in DNA methylation status between a certain type of body fluid and other types of body fluids and to have methylation or unmethylation pattern only in a particular type of body fluid. From further locus-specific methylation analysis in additional samples, 1 to 3 CpG sites were selected for each body fluid. Then, a multiplex methylation SNaPshot reaction was constructed to analyze methylation status of 8 body fluid-specific CpG sites. The developed multiplex reaction positively identifies blood, saliva, semen and the body fluid which originates from female reproductive organ in one reaction, and produces successful DNA methylation profiles in aged or mixed samples. Although it remains to be investigated whether this approach is more sensitive, more practical than RNA- or peptide-based assays and whether it can be successfully applied to forensic casework, the results of the present study will be useful for the forensic investigators dealing with body fluid samples.

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1. Introduction

Besides providing information on donor identity, body fluids found at the crime scenes play an important role in forensic investigations [1]. Determination of the type and origin of body fluids can provide important clues for crime scene reconstruction by linking donors and actual criminal acts. However, most currently used catalytic, enzymatic, and immunologic tests for forensic body fluid identification suffer from several limitations, such as low specificity, lack of sensitivity, sample destruction, instability of biomolecules being assayed or incompatibility with downstream individual identification [2].

In recent years, tissue-specific RNA expression and DNA methylation have been suggested as a promising new tool to overcome these limitations and to distinguish between different types of body fluids [3–18]. Especially, recent approaches based on tissue-specific mRNA and miRNA expression have proven to be useful because of their high specificity, ability to be analyzed in multiplex, and unexpected long-term stability [3–11]. However, particular care and effort to inactivate ubiquitously present ribonucleases still prevents forensic experts from routinely integrating RNA analysis into a forensic casework scheme. On the other hand, the DNA methylation-based method uses the same biological source of DNA for personal identification profiling; thus, special training is not required for an assay. Moreover, body fluid-specific differential DNA methylation showed high specificity and multiplex analysis was also possible [13–18]. Nevertheless, there is a need to identify more body fluid-specific DNA methylation markers. Many semen-specific markers have been identified, but markers specific to blood, saliva, vaginal fluid, menstrual blood, or skin have been reported only in a few publications and require more validation [12,14,18].

Meanwhile, rapid advances in epigenetics make genome-wide DNA methylation profiling accessible to many researchers. For example, Illumina's HumanMethylation450 BeadChip array provides DNA methylation profiles at more than 450,000 CpG sites using only a small amount of DNA. Therefore, DNA methylation profiling for various types of body fluids using this technique would enable the identification of significant DNA methylation markers that are useful for forensic body fluid identification.

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Therefore, we produced genome-wide DNA methylation profiles of blood, saliva, semen, vaginal fluid and menstrual blood using Illumina's HumanMethylation450 BeadChip array and selected several body fluid-specific DNA methylation marker candidates. Then, we developed a multiplex methylation SNaPshot to analyze DNA methylation profiles at the selected body fluid-specific CpG sites. Body fluid specificity and forensic applicability of the multiplex system were also tested with various samples.

2. Materials and methods

2.1. Samples

Blood, saliva, and semen from 84 males and blood, saliva, vaginal fluid, and menstrual blood from 16 female volunteers were collected using procedures approved by the Institutional Review Board of Severance Hospital, Yonsei University in Seoul, Korea. The 100 donors provided written informed consent after the goals, and procedures of the study were explained. Blood was collected in a syringe, and 200 μL aliquots were stored frozen. Saliva and freshly ejaculated semen were collected in a microcentrifuge tube and plastic cups, respectively, and 200 μL aliquots of each were frozen for storage. Vaginal fluid and menstrual blood were collected using sterile cotton swabs and allowed to dry at room temperature. Dried swabs were stored frozen until use.

DNA was extracted from each aliquot of blood, saliva, and semen using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) and from each swab of vaginal fluid and menstrual blood using a QIAamp® DNA Investigator Kit (QIAGEN) following the manufacturer's instructions. Extracted DNA was quantified using a Quantifiler® Duo DNA Quantification Kit (Applied Biosystems, Foster City, CA, USA).

2.2. Genome-wide DNA methylation profiling using HumanMethylation450 BeadChip array

Microarray hybridization was performed by Macrogen, Inc. (Seoul, Korea). One to two micrograms of genomic DNA obtained from 42 samples, including 12 samples each of blood, saliva and semen, and 3 samples each of vaginal fluid and menstrual blood, were bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Bisulfite-converted DNA was whole-genome amplified using the MSM master mix (Illumina, San Diego, CA, USA) and incubated at 37 °C for 22 h. Amplified DNA was then fragmented and hybridized to HumanMethylation450 BeadChips (Illumina) in an Illumina Hybridisation Oven at 48 °C for a minimum of 16 h. Following hybridization, single base extension of hybridized DNA was performed using hapten-modified nucleotides. Staining was then processed by adding fluorescently labeled antibodies in several steps to amplify the signals, and the arrays were washed. The image of the stained BeadChip was scanned with an iScan Reader (Illumina), and β-values were extracted using GenomeStudio software v. 2011 (Illumina). Probe sets with signal intensities under the average background for negative control probes (detection *P*-values ≥ 0.05) were removed from the data set. In addition, probe sets that had greater than 25% missing values were removed from the data set. The calculated β-score corresponds to the percentage methylation value at a specific CpG site and varied between 0 and 1. To adjust β-scores for batch effects among BeadChips, probe sets with missing values were removed, and β-scores were normalized by nonparametric empirical Bayes framework method using ComBat within an R package called Surrogate Variable Analysis (http://www.bioconductor.org/packages/release/bioc/html/sva.html).

To identify CpGs with body fluid-specific differential DNA methylation, the mean value of the average β-scores was calculated for every CpG in each type of body fluid, and they were compared among the body fluids. The CpGs that exhibited more than 30% discrepancy in the mean values of average β-scores between a certain type of body fluid and the other types of body fluids with a *P*-value < 0.05 in a Student's *t*-test were selected as body fluid-specific CpG marker candidates. Then, CpGs that exhibited body fluid-specific complete methylation or unmethylation pattern were further extracted for the following analyses.

2.3. Targeted bisulfite sequencing using direct sequencing and methylation SNaPshot

The body fluid specificity of selected CpGs was further investigated using direct sequencing and a single-base extension reaction (SBE) of bisulfite-converted DNA in 2 samples from the array and 2 additional samples for each body fluid. Bisulfite sequencing was designed using in silico-bisulfite-converted genomic reference sequences as determined by beadchip results. PCR primers for the amplification of bisulfite-converted genomic DNA were designed using the Methprimer program (http://www.urogene.org/methprimer/index1.html) [19] or the Primer 3 program (http://bioinfo.ut.ee/primer3/), and SBE primers for the target CpGs within the PCR products were designed using the Batchprimer3 program (http://wheat.pw.usda.gov/demos/BatchPrimer3/) [20].

PCR was performed in 20 μL reactions containing 1 μL of bisulfite-converted DNA, 1 U of AmpliTaq Gold® DNA Polymerase, 2 μL of Gold ST*R 10 × Buffer, and 0.4–1.0 μM of each primer. Bisulfite-converted DNA was obtained by modification of 100 ng genomic DNA using the Imprint® DNA Modification Kit (Sigma–Aldrich Inc., St. Louis, MO, USA) according to the manufacturer's protocol. PCR cycling was conducted in a PTC-200 DNA engine under the following conditions: 95 °C for 11 min; 34 cycles of 94 °C for 20 s, 56 °C for 60 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. Then, 5 μL of PCR products were purified with 1 μL of ExoSAP-IT (USB, Cleveland, OH, USA) by incubation at 37 °C for 45 min followed by heat inactivation at 80 °C for 15 min. Purified PCR products were sequenced using one of forward or reverse primers and BigDye® Terminator v3.1Cycle Sequencing Kit (Applied Biosystems). Sequencing results were analyzed in an ABI 3730xl DNA Analyzer (Applied Biosystems), and the sequencing data were aligned against in silico-converted genomic reference sequences using BiQ Analyzer (http://biq-analyzer.bioinf.mpi-sb.mpg.de/) [21].

For the CpG sites that showed body fluid-specific DNA methylation change in direct sequencing of bisulfite converted DNA, an SBE reaction was performed using 1 μL of purified PCR products, 0.2–0.4 μM of SBE primers and a SNaPshot™ Kit (Applied Biosystems) according to the manufacturer's instructions. Extension products were analyzed using an ABI PRISM 3130 Genetic Analyzer and GeneScan software 3.1 (Applied Biosystems). Based on the SBE results, CpGs that showed body fluid-specific methylation change in a particular type of body fluid with complete methylation or unmethylation pattern in other types of body fluids were preferentially selected for the design of a multiplex methylation SNaPshot reaction.

2.4. Multiplex methylation SNaPshot for body fluid identification

Multiplex methylation SNaPshot was designed to quantify DNA methylation simultaneously at multiple CpG sites. PCR primers for the amplification of bisulfite-converted genomic DNA and SBE primers for the target CpGs within the PCR products are shown in Table S1. PCR amplification and SBE reaction were carried out with

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