Evaluation of the usefulness of saliva for DNA methylation analysis in cohort studies

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Abstract

Introduction: Epigenetic information such as DNA methylation is a useful biomarker that reflects complex gene-environmental interaction. Peripheral tissues such as blood and saliva are commonly collected as the source of genomic DNA in cohort studies. Epigenetic studies mainly use blood, while a few studies have addressed the epigenetic characteristics of saliva.

Methods: The effects of methods for DNA extraction and purification from saliva on DNA methylation were surveyed using Illumina Infinium HumanMethylation450 BeadChip. Using 386,661 probes, DNA methylation differences between blood and saliva from 22 healthy volunteers, and their functional and structural characteristics were examined. CpG sites with DNA methylation levels showing large interindividual variations in blood were evaluated using saliva DNA methylation profiles.

Results: Genomic DNA prepared by simplified protocol from saliva showed a similar quality DNA methylation profile to that derived from the manufacturer provided protocol. Consistent with previous studies, the DNA methylation profiles of blood and saliva showed high correlations. Blood showed 1,514 hypomethylated and 2099 hypermethylated probes, suggesting source-dependent DNA methylation patterns. CpG sites with large methylation difference between the two sources were underrepresented in the promoter regions and enriched within gene bodies. CpG sites with large interindividual methylation variations in blood also showed considerable variations in saliva.

Conclusion: In addition to high correlation in DNA methylation profiles, CpG sites showing large interindividual DNA methylation differences were similar between blood and saliva, ensuring saliva could be a suitable alternative source for genomic DNA in cohort studies. Consideration of source-dependent DNA methylation differences will, however, be necessary.

Keywords
biomarker, blood, epigenetics, interindividual variation
1 | INTRODUCTION

DNA methylation is a reversible chemical modification of cytosine residue in the DNA sequence and is an important regulator of gene expression. It reflects the genetic background of individuals, as well as environmental factors.\(^1\) DNA methylation is therefore closely associated with neuropsychiatric disorders\(^2\) and is an informative biological marker in cohort studies.\(^3\,\text{4}\,\text{5}\)

In epidemiological cohort studies, peripheral tissues such as blood and saliva are commonly used as the source of genomic DNA. Because cohort studies usually deal with hundreds to thousands of subjects in a longitudinal manner, saliva has clear advantages over blood because it is noninvasive, easy to use, and does not require trained medical professionals for sample collection.\(^6\) However, saliva has been used less often than blood in epigenetic studies, and only a few studies have addressed the epigenetic characteristics and uniqueness of saliva.\(^7\,\text{8}\,\text{9}\)

When conducting large-scale epigenome-wide association studies (EWAS), a previous study has demonstrated that CpG sites whose DNA methylation levels show large interindividual variations are useful for identifying disease-related epigenetic changes.\(^9\) That study used blood data to evaluate the CpG sites, and the validity of using saliva needs to be addressed.

In this study, we investigated the usefulness of saliva samples for providing epigenetic data in cohort studies. First, we compared the methods of DNA extraction and purification from saliva using the commercially available kit. Second, we identified differentially methylated CpG sites between blood and saliva, and revealed their characteristics. Third, we tested whether the CpG sites showing large interindividual methylation differences in blood\(^9\) also showed variations in saliva.

2 | MATERIALS AND METHODS

2.1 | Subjects

To evaluate DNA extraction and purification methods, saliva samples were collected from 3 Japanese females. To compare the DNA methylation profiles of blood and saliva, we collected blood and saliva from 22 age-matched healthy volunteers (male: mean age 31.1 ± 4.9, N = 15; female: 29.7 ± 6.8, N = 7). This study conformed to the provisions of the Declaration of Helsinki. The ethics committees of the University of Tokyo Hospital and collaborative research organizations approved this study.

2.2 | Genomic DNA extraction

From each participant, we obtained 2 mL of saliva using the Oragene-DNA collection kit\(^10\) (DNAgenotek Inc., Ontario, Canada). Genomic DNA was extracted according to the protocol of the prepIT-C2D Genomic DNA MiniPrep kit (DNAgenotek Inc.). The protocol contained a step to purify DNA using the MiniPrep column and another step of RNA degradation using RNase A. To evaluate the DNA extraction and purification methods, we compared four protocols: (1) employing the MiniPrep column for DNA purification and RNase A (the manufacturer’s protocol without modification); (2) employing the MiniPrep column without using RNase A; (3) employing ethanol precipitation instead of the MiniPrep column and using RNase A; and (4) employing ethanol precipitation without using RNase A. Genomic DNA extraction from the saliva of 22 subjects was performed using protocol (1). Genomic DNA extraction from blood was performed using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

2.3 | DNA methylation analysis and data analysis

We analyzed DNA methylation using Infinium HumanMethylation450 BeadChip (Illumina, CA, USA) according to the manufacturer’s instructions. The assay contained >485 000 CpG targets and covered 99% NCBI Reference Sequence genes. DNA methylation data were processed under R environment.\(^11\) Color normalization was performed using the background correction and internal control probes included in each chip. We excluded probes that (1) showed detection P value ≥.05; (2) were located on X or Y chromosomes; (3) had potential SNPs; (4) might cross-hybridize with unspecified genomic regions;\(^12\) and (5) lacked data for at least one sample. Differentially methylated probes were defined as P value <.05 by the Wilcoxon signed-rank test with a difference of mean β value between blood and saliva larger than 0.2. Gene ontology (GO) analysis was performed using PANTHER,\(^13\) applying Bonferroni correction. Principal component analysis (PCA) and hierarchical clustering analysis were conducted using the maptools package in R. The CpG sites showing large interindividual methylation variation in blood were retrieved from a previous report.\(^9\) According to the definition,\(^9\) we used CpG sites whose reference interval (RI) was larger than 30. Correlation between standard deviation (SD) of DNA methylation values in this study and RI in the previously identified CpGs was calculated using Spearman’s rank correlation. Preference for genomic region of the differentially methylated probes was assessed using Fisher’s exact test.

3 | RESULTS AND DISCUSSION

We performed Illumina Infinium HumanMethylation450 BeadChip assay using genomic DNA derived from the saliva of three subjects. For each subject, we performed 4 patterns of DNA extraction and purification, including the protocol provided by the manufacturer. The protocol included column purification followed by RNase A treatment, and we performed either column purification or ethanol precipitation with or without RNase A treatment. Evaluation of data quality was made by comparing the (1) total number of detected probes at the levels of detection P value; (2) average intensities of the array probes; and (3) PCA. The comparisons revealed that there were no statistically significant differences in the total number of detected probes or the intensities of the probe signals (ANOVA, P > .05) (Figure 1A). In addition, PCA showed sample-dependent,
terms such as cell periphery, immune system, and plasma membrane. 

Blood, associated with 574 genes, and 2099 hypomethylated probes were further analyzed. Both clustering analysis and PCA showed that the DNA methylation profile of saliva was clearly separated from that of blood (Figure S1 A). However, as expected, the average DNA methylation profiles showed a high correlation (R = 0.977) between the two sample sources, indicating a close relationship between them (Supplementary Figure S1 B). Age and sex would be important factors for DNA methylation status at the specific genomic regions. However, our PCA and clustering analysis suggested that the overall DNA methylation profiles were not affected by age or sex. Therefore, we did not consider the effect of these factors in detail in this study. We then attempted to estimate what constituted the main cell population of our saliva samples. We calculated the correlation of the overall DNA methylation level with the publicly available data from samples separated into several blood cell lineages. Our saliva data showed best correlation with granulocytes (R = 0.947). However, our saliva samples did not separate according to specific blood cell lineages by clustering analysis and PCA, likely due to differences in the races, ages, and experimental batches (data not shown).

Based on the statistical analysis and the extent of DNA methylation difference, we identified 1514 hypomethylated probes in blood, associated with 574 genes, and 2099 hypomethylated probes in saliva, associated with 1117 genes. GO analyses revealed that GO terms such as cell periphery, immune system, and plasma membrane commonly appeared in both sources (Figure 2A). Notably, GO terms related to leukocytes were found in the top list of hypomethylated genes in blood, and those related to enzyme binding and vesicle-mediated transport were found in the top list of hypomethylated genes in saliva, likely reflecting that upregulated genes in each source were hypomethylated in that source. Compared with the previous study, which examined differential DNA methylation profiles between peripheral whole blood and saliva using HumanMethylation27 BeadChip, GO terms such as plasma membrane and immune system process are commonly appeared. However, those related to regulation of signaling and cell communication were not detected in the previous report. This difference may be due to the differences in the age and race of subjects and type of array platform. We next examined the genomic context of the differentially methylated probes. We found that differentially methylated probes were underrepresented in the CpG island and promoter regions (TSS1500 and TSS200) and were enriched in the 5'-UTR and gene body (Figure 2B). Overall, these observations were generally consistent with those of previous studies.

We also inspected in detail those genes showing DNA methylation differences in blood and saliva (Table S1). Genes such as TBX1, S1PR4, and SPEG were hypomethylated in blood, unlike saliva. TBX1 encodes a transcription factor involved in development and reported to be related to heart disease and DiGeorge syndrome. S1PR4 encodes a G-protein-coupled receptor important for immune response. SPEG encodes a protein similar to the myosin light chain kinase family, which is important for cytoskeletal and cardiovascular development. On the other hand, RIN2, DOT1L, and BZRAP1 showed hypomethylation in saliva. RIN2 encodes a protein that works as a guanine nucleotide exchange factor for RAB5, and its mutations are known to cause several syndromes related to defects in connective tissue. DOT1L encodes a histone methyltransferase with important functions in cartilage and
blood vessel homeostasis. BZRAP1 encodes a protein forming benzodiazepine receptor complex in mitochondria.

Hachiya and colleagues previously identified CpG sites where DNA methylation levels showed large interindividual differences in blood cells from the whole genome bisulfite sequencing data. They also showed that such CpG sites increased the efficacy in detecting differential DNA methylation in EWAS. To evaluate the usefulness for saliva of the CpG sites previously identified in blood, we compared the variance of DNA methylation levels in our data with that of the previous study. We used 23,076 probes that were available in both our data set and previous data set. As expected, we observed a positive correlation between our blood data ($R = 0.323, P < 2.2 \times 10^{-16}$), suggesting that saliva does reflect the interindividual DNA methylation profiles identified in blood.

In conclusion, saliva can be used as an alternative to blood in DNA methylation analysis for cohort studies. The informative CpG sites to be examined in the cohort study are generally common between the two sources. However, a group of CpG sites showed saliva-dependent DNA methylation profiles, so target CpG sites should be carefully designed.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
M.B., K.K., and K.I. designed the research. S.K., S.F., and M.B. collected samples and performed experiment. Y.M., A.F., S.K., T.I., Z.Z., and S.J. analyzed the data. Y.N. managed the data. Y.M., A.F., and K.I. prepared the manuscript.

DATA ACCESSIBILITY
Raw data have been deposited in Gene Expression Omnibus and are accessible through GSE130153.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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