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Genome-wide DNA Methylation Profiling of Blood from Monozygotic Twins Discordant for Myocardial Infarction

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Abstract. *Background/Aim: This study aimed to measure the DNA methylation state of thousands of CpG islands in the blood of two monozygotic twins that were discordant for cardiovascular disease (CVD). Twin 1 had suffered myocardial infarction, while the other was healthy. Patients and Methods: Since the aim of this study was to identify differentially methylated regions which might act as potential markers, reduced-representation bisulfite libraries were used for whole-genome methylation analysis. Results: According to the analysis, 11 genes lipid droplet associated hydrolase (LDAH), apolipoprotein B (APOB), acyl-CoA synthetase medium chain family member 2A (ACSM2A), acyl-CoA synthetase medium chain family member 5(ACSM5), acyl-CoA synthetase family member 3 (ACSF3), carboxylesterase 1 (CES1), carboxylesterase 1 pseudogene 1 (CES1P1), AFG3 like matrix AAA peptidase subunit 2 (AFG3L2), iron-sulfur cluster assembly enzyme (ISCU), SEC14 like lipid binding 2 (SEC14L2) and microsomal triglyceride transfer protein (MTTP) were all hypomethylated in DNA from twin 2, the unaffected twin. Methylation changes were observed at different multiple loci between the twins, suggesting loci that are affected by disease status in identical genetic backgrounds. Conclusion: This twin study may contribute significantly to the understanding of the genetic basis of CVD and resulting myocardial infarction. This approach may allow identification of possible target loci associated with aberrant epigenetic regulation in CVD.*

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Cardiovascular disease (CVD) is a cause of worldwide mortality. In addition, diabetes mellitus, hypercho-lesterolemia, smoking, hypertension, obesity and physical inactivity are primary risk factors for CVD (1, 2). Factors of CVD include age, male gender, ethnicity and family history (3). Genetic factors also play a key role in CVD. Genome-wide association studies (GWAS) have identified multiple genes and single nucleotide polymorphisms (SNPs) involved in CVD (4). However, most of these loci only increase the risk of CVD modestly, and other studies have sought epigenetic factors that might be associated with disease incidence and risk.

Specifically, recent advances in the field of epigenetics have led to the investigation of DNA methylation and its association with manifestations of disease phenotype. Alterations in DNA methylation mediate underlying CVD risk (5). For example, Epigenome-wide association studies investigated regions of methylated DNA associated with phenotypes and identified gene regions that are significantly associated with risk factors for CVD such as high body mass index, high blood lipid levels, and type 2 diabetes (6-8). It also found the DNA methylation status in blood samples to be associated with CVD itself (9).

It is known that not all genes are expressed at the same time by all cell types (10). Differences in gene-expression profiles in cells and tissues occur due to epigenetic mechanisms. CpG islands are stretches of DNA roughly 1000 base pairs long that have a higher CpG density than the rest of the genome but often are not methylated (10). CpG islands contain roughly 70% of gene promoters (11). The promoter regions for housekeeping genes are often embedded in CpG islands (12). CpG islands, especially those associated with promoters, are highly conserved between mice and humans (13). The location and preservation of CpG islands throughout evolution implies that these regions possess a functional importance.

Here we used reduced-representation bisulfite sequencing (RRBS) to measure the DNA methylation state of thousands of CpG islands in the blood of two monozygotic twins that were discordant for CVD. This approach may allow identification of possible target loci associated with aberrant epigenetic regulation in CVD.

Patients and Methods

Design and study subjects. A 27-year-old male presented to our outpatient clinic for further evaluation of premature coronary artery disease. He had undergone a primary percutaneous coronary intervention for an anterior myocardial infarction (MI) 1 month earlier. The diagnosis of MI had been based on typical electrocardiographic changes and increased serum activities of enzymes including creatine kinase, aspartate aminotransferase, and lactate dehydrogenase; it was confirmed by the presence of a wall motion abnormality on left ventriculography and attendant stenosis of the major coronary arteries. It was successfully treated with a stent implantation in the proximal left anterior descending artery. He was asymptomatic and with normal physical examination findings. On echocardiography, his left ejection fraction was preserved (55%).

His monozygotic twin was also investigated along with the patient given the potential catastrophic results of the disease. They were both soldiers, with moderate daily activity. The medical history was unremarkable, with no evident risk factors. They were also screened for non-traditional risk factors, which yielded no significant results. The absence of coronary artery disease was confirmed by multislice computed tomography imaging in the unaffected twin. Both twins were asymptomatic at the 24-month clinical follow-up.

Sample collection and DNA extraction. Blood samples were collected in EDTA vacutainers at Pamukkake University Medical faculty, Department of Cardiology. Written informed consent was obtained. Blood cell counts and biochemical tests were carried out (Table I). Genomic DNA was isolated from the individuals by standard phenol-chloroform extraction method. This study was approved by the Ethics Committee of Pamukkale University Faculty of Medicine (ethical approval number 60116787-020/49148).

DNA methylation assay. Genomic DNA was isolated by standard phenol-chloroform extraction method and used as input to prepare RRBS libraries as described previously (14) with minor modifications. For each sample, 50-100 ng of purified genomic DNA was digested with 20 U of *Msp*I (NEB, USA) at 37˚C o/n in the presence of RNase Cocktail Mix (Ambion, USA). End-repair and dA-tailing was performed by the addition of Klenow Fragment 3'->5' exo- (NEB) in the presence of dATP, dGTP and d5mCTP (Fermentas, USA). Adapter ligation was performed by the addition of 0.3 μl of Illumina TruSeq methylated Adapters (Illumina, TruSeq Nano, USA) and 2 μl of Illumina Ligation Mix 2 (Illumina, TruSeq Nano). Samples were pooled and purified using an equal volume of SPRI beads (Beckman Coulter, USA). Size-selection was performed using SPRI beads to enrich for fragments from 200 to 300 bp. Bisulfite treatment was performed using Epitect Bisulfite kit (QIAGEN, USA) according to the manufacturer's protocol, except that two consecutive rounds of conversion were performed, for a total of 10 h of incubation. Purified converted DNA was polymerase chain reaction (PCR) amplified using MyTaq HS Mix (Bioline, USA) and TruSeq PCR Primer Cocktail (Illumina, TruSeq Nano, USA) according to the following protocol: Initial denaturation at 98˚C for 30 s; 12 cycles of 98˚C for 15 s, 60˚C for 30 s, 72˚C for 3 0 s; final extension at 72˚C for 5 min. Amplified libraries were purified twice with an equal amount of SPRI beads to remove primer and adapter dimers. Libraries were sequenced 100 bp single-end on an Illumina HiSeq4000.

Differential methylation analysis. Reads were aligned to the reference genome (GRCh37) using BS-Seeker2 (15). Methylation levels were called using the default parameters of BS-Seeker2. CpG

Table I. *Hematological and biochemical data of the twins.*

	Twin-1 (case)	Twin-2
Age, years/gender	27/Male	27/Male
BMI, kg/m^2	24	24.5
WBC, $\times 10^3$/μl	7.13	7.42
RBC, $\times 10^{12}$/l	5.9	6.42
Hemoglobin, g/dl	17.2	18.3
Hct, %	49.6	52.6
MCV, fl	84	81.9
MCH, g/dl	29.1	28.5
MCHC, pg	34.7	34.9
RDW, %	13.9	12
platelet, k/μl	221	252
PDW, %	66.1	41.2
glucose, mg/dl	85	101
LDL cholesterol	78	179
Urea, mg/dl	32	36
BUN, mg/dl	15	17
Creatinine, mg/dl	0.94	1.01
Sodium, mmol/l	136	141
Potassium, mmol/l	4.12	4.88
AST, IU/l	18	34
ALT, IU/l	34	86
uric acid, mg/dl	5.9	7.8
HDL cholesterol, mg/dl	40	36
Total cholesterol, mg/dl	138	251
Triglyceride, mg/dl	101	179
VLDL cholesterol, mg/dl	20	36
von Willebrant factor antigen, %	194.5	204.6
Anti-phosfolipid IGM	2.1	2.2
Anti-phosfolipid IGG	2.8	2.17
Anti-cardiolipin IGM	2.79	2.17
Anti-cardiolipin IGG	5.86	3.48
Sedimentation 1st hour	15	22
Active protein C resistance	2.62	2.8
Lupus anticoagulant	0.96	0.98

BMI: Body mass index; WBC: white blood cells; RBC: red blood cells; Hct: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; PDW: platelet distribution width; LDL: low density lipoprotein; BUN: blood urea nitrogen; AST: aspartate aminotransferase; ALT: alanine aminotransferase; HDL: high-density lipoprotein; VLDL: very-low-density lipoprotein; IGM: immunoglobulin M; IGG: immunoglobulin G.

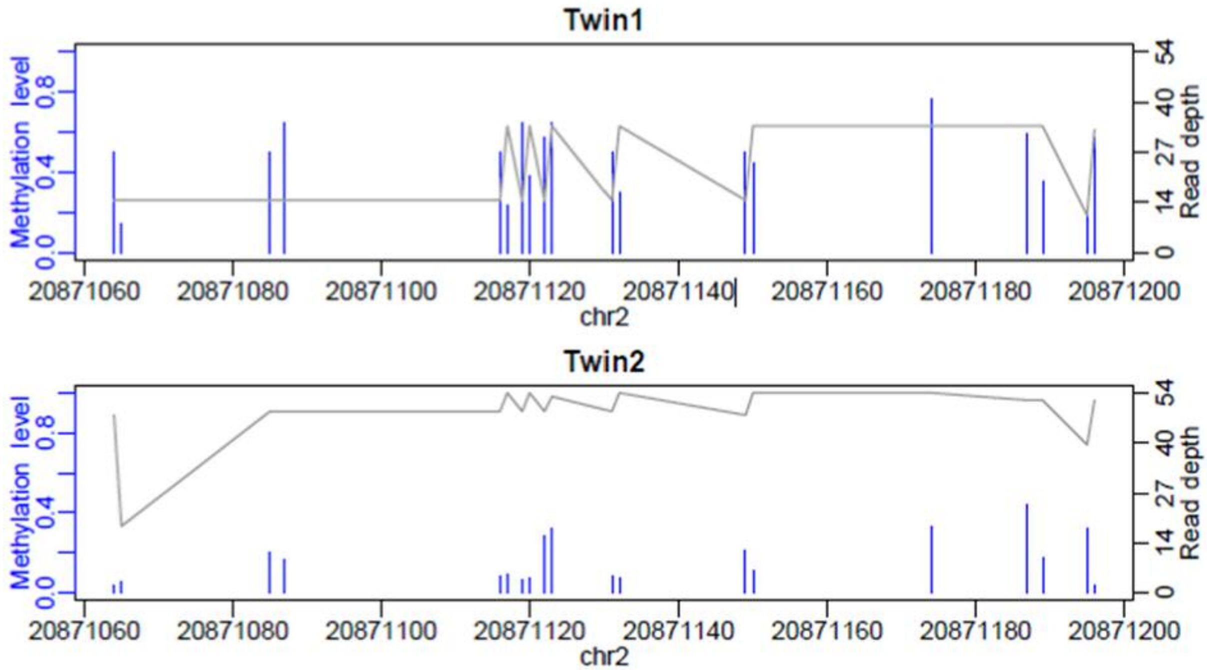

Figure 1. *Differentially methylated loci in twins 1 (case) and 2 (healthy). chr: Chromosome.*

sites with coverage more than 10 were retained for the downstream analysis. DSS, an R package, was used to determine the differentially methylated loci (DML). A total of 3,004 out of 2,562,092 methylated loci were called as DML with a *p*-value cutoff of 0.01.

Enrichment analysis. To associate the DML with genes, a regulatory region was first assigned to a gene. Following a similar strategy as that used in the program GREAT (16), each gene was assigned a basal regulatory domain of 5 kb upstream and 1 kb downstream of the transcription starting site. The gene's regulatory domain was then extended in both directions to the nearest gene's basal domain but no more than 1000 kb in one direction. The DML and the background methylated sites were then overlapped with the genes' regulatory regions. Following this approach, each gene's regulatory region was assigned to a number of DML and a number of background methylated sites. A hypergeometric test was then performed for each gene to estimate whether the number of DML was higher than that expected by chance. The Benjamini–Hochberg procedure was used to control for the false-discovery rate. Using this procedure, 480 genes were retained at a false-discovery rate level of 0.01. These genes had regulatory regions that were considered differentially methylated between samples. Enrichment analysis was performed for these genes using Enrichr (17).

Cell type percentage estimation. The CGmap files output from BS-Seeker2 was input for CELLFi. Purified B-cell, T-cell, monocyte, neutrophil and natural killer cell methylation data were used as the reference, the other parameters were set as default. The percentages were output from CELLFi and used to prepare the graphical representation of cell type composition for each twin. The detailed method can be found in (17).

Results

Clinical data. Table I shows hematological and biochemical data of the twins, which reveal that some of the healthy twin's (twin 2) lipid parameters were higher than those of the case (twin 1). Since twin 1 was treated with aspirin and statins (for 2 months), lipid levels were lower than those of twin 2. Thus, the statins may explain part of the difference in the laboratory data rather than there being a solely epigenetic influence, considering the short amount of time between the index event, myocardial infarction, and tests performed.

Differential methylation analysis. DNA from the twins was used to prepare RRBS libraries as described previously with minor modifications (14). The libraries were sequenced using an Illumina HiSeq4000 yielding more than 20 million reads for each twin. After sequencing, the reads were aligned and methylation called using BS-Seeker2 (15). We selected CpG sites that had coverage of at least 10 in both twins, resulting in 2,562,092 CpG sites. We then used DSS, an R package, to determine DML and found 3,004 DML by selecting a *p*-value threshold of 0.01. We show an example region that contained several DML (Figure 1). This region is close to the Lipid droplet associated hydrolase (*LDAH)* gene (13 kb), which plays a role in cholesterol and lipoprotein metabolism.

Term enrichment analysis of differentially methylated genes. To find the genes associated with the DML, we first assigned a regulatory region for each gene (see Materials and Methods). Then we overlapped the gene's regulatory region with the DML and all of the loci that were measured in both twins. To determine which gene's regulatory region was differentially methylated, a hypergeometric test was used for each gene and a false-discovery rate of 0.01. This process resulted in 480 genes, which were then used for enrichment analysis. Eleven genes, namely, lipid droplet-associated hydrolase (*LDAH*), apolipoprotein B (*APOB*), acyl-CoA synthetase medium chain family member 2A (*ACSM2A*), acyl-CoA synthetase medium chain family member 5 (*ACSM5*), acyl-CoA synthetase family member 3 (*ACSF3*), carboxylesterase 1 (*CES1*), carboxylesterase 1 pseudogene 1 (*CES1P1*), AFG3-like matrix AAA peptidase subunit 2 (*AFG3L2*), iron-sulfur cluster assembly enzyme (*ISCU*), SEC14 like lipid binding 2 (*SEC14L2*) and microsomal triglyceride transfer protein (*MTTP*) were found to be involved in fatty acid and cholesterol metabolism, and were all hypomethylated in DNA from twin 2. This indicates these genes might be highly expressed in twin 2 and potentially explains why twin 2 had high triglyceride and cholesterol levels. The enrichment analysis result of Gene Ontology (GO) molecular function is shown in Figure 2, from which it can be seen that these genes were enriched for fatty acid ligase activities.

Cell type prediction using the methylation data. To study whether the observed DNA methylation differences were associated with different cell type composition, we performed cell type deconvolution using the methylation data. CELLFi, an unpublished tool developed in the laboratory, was used to estimate the percentage of each reference cell type. CELLFi uses CpG methylation calls from purified reference samples to perform cell mixture deconvolution of heterogenous samples. Using a non-negative least squares regression, the tool was used to estimate the fractional methylation contribution of each reference cell type, namely B-cells, T-cells, monocytes, neutrophils and cells, for each twin. The results are shown in Figure 3. We observed a slight difference in cell type composition, but the differences were not large, suggesting that differences in cell type composition do not explain the DNA methylation differences we observed.

Discussion

Lifestyle factors for cardiovascular diseases have a significant impact on the development of the disease. Genome-wide studies reveal the effects of altered gene expression. For example, Brahmachari *et al.* reported the identification of regions across the genome that are differentially methylated in CVD (18). In recent years, research on cardiovascular epigenetics has begun to expand rapidly from biological and animal studies to epidemiological studies. In studies of blood samples, CVD has been associated with methylation of repetitive sequences such as long-interspersed nucleotide repeating elements-1 (*LINE1*) and *ALU* elements (19, 20). Numerous epidemiological studies have shown that lifestyle and environmental factors may affect cardiovascular health of individuals and populations (19-21).

Epigenetic mechanisms may play a role in the development of CVD. This is of particular interest within the framework of the developmental origins of risk factors that occur during fetal life, such as maternal exposure (22). As mentioned, epigenetic mechanisms are crucial during development of the organism. The *in-utero* period therefore represents a vulnerable time frame during which external stimuli can have considerable influence on long-term risks (22, 23).

In the present study, we identified the differentially methylated regions between monozygotic twins discordant for CVD; one (twin 1, 27 years old) of suffered MI while the other was healthy. Since the aim of this study was to identify differentially methylated regions which might act as potential markers, we used RRBS for whole-genome methylation analysis. According to the analysis, 11 genes (*LDAH, APOB, ACSM2A, ACSM5, ACSF3, CES1, CES1P1, AFG3L2, ISCU, SEC14L2* and *MTTP*) were all hypomethylated in DNA from twin 2, the unaffected twin. These genes are involved in fatty acid and cholesterol metabolism. The fact that they were hypomethylated in the unaffected twin suggests that their gene expression may also be higher in this individual, which is concordant with his higher lipid levels. As the affected twin was treated with statins to reduce the cholesterol level, it is possible that some of the observed epigenetic differences might have resulted from this drug treatment. However, we cannot conclude from this that the hypermethylation of these loci, possibly resulting from statin treatment, is causal for myocardial infarction. To test this hypothesis, twin studies with larger cohorts would be needed (24).

Although twin studies have contributed significantly to the understanding of the genetic basis of coronary artery disease and resulting myocardial infarction, this study design was not utilized for the GWAS era as monozygotic twins are genetically identical and dizygotic twins are not different from ordinary siblings (25). By contrast, disease-discordant monozygotic twins, who are completely matched for genetics, age, sex, cohort effects, maternal influences and common environment, and are closely matched for other lifestyle factors, are ideal for detecting epigenetic differences that may underlie their discordant traits. While this study examined only a single such pair, the significant epigenetic differences in lipid pathways that we observed warrant future investigation in larger cohorts (26).

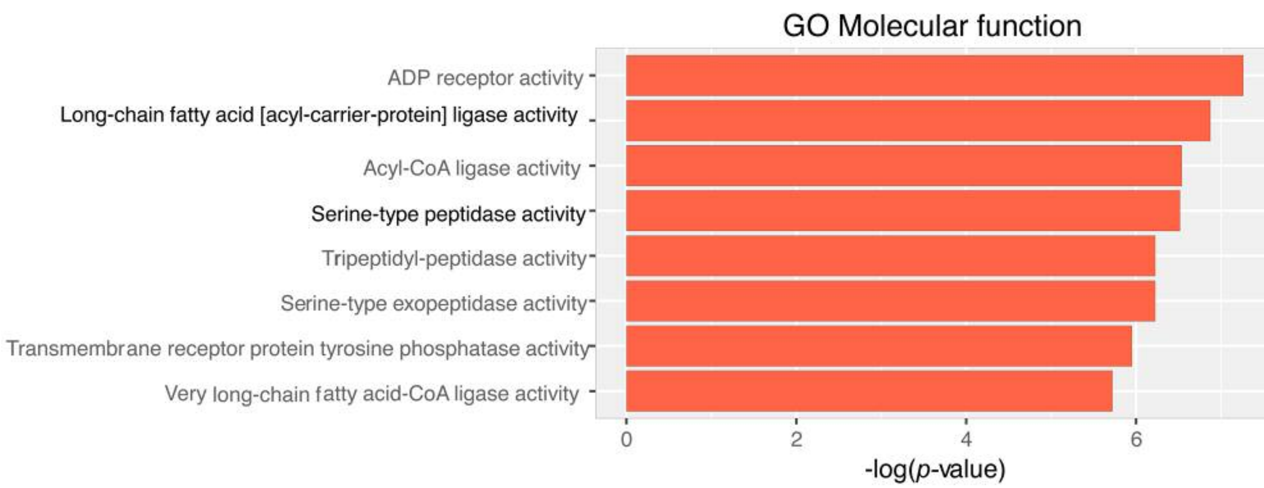

Figure 2. *Enrichment analysis result of Gene Ontology.*

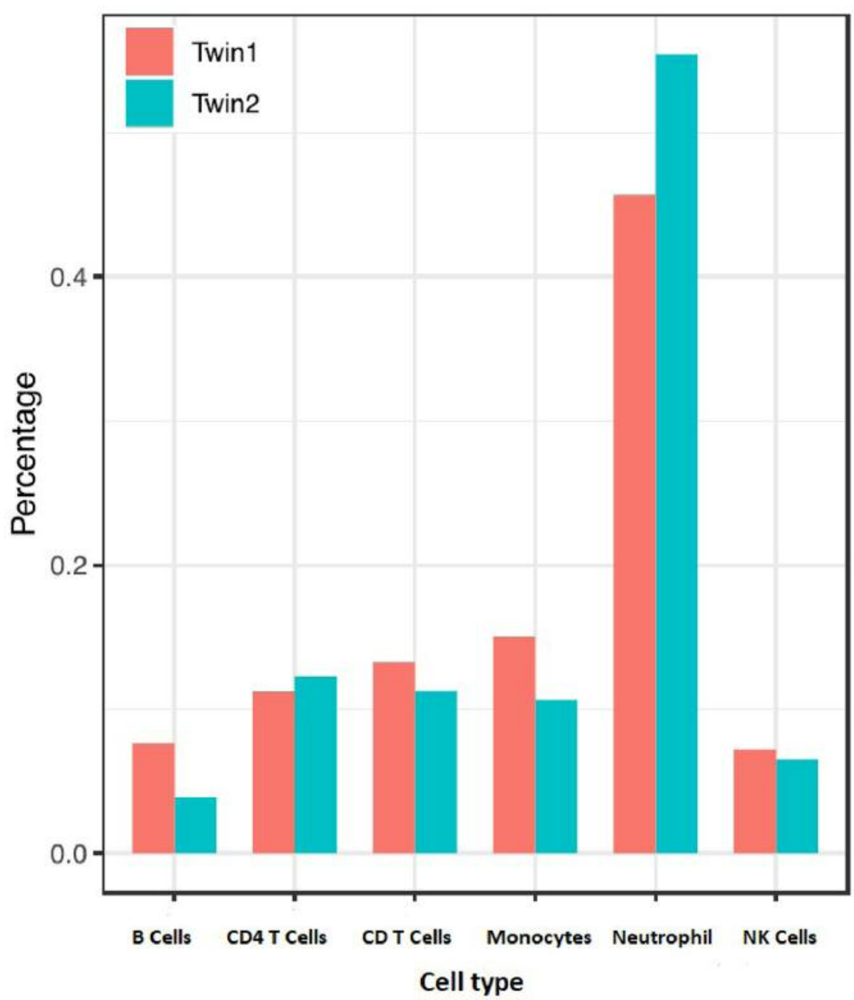

Figure 3. *Estimated cell type composition for each twin. NK: Natural killer.*

Conflicts of Interest

The Authors declare no competing interests in regard to this study.

Authors' Contributions

M.P. and A.K. designed the study. I.D.K and O.K. analyzed clinical data. M.M., F.M. and A.K. performed experiments and also contributed ideas and insights. M.P., A.K., F.M, and M.M. wrote the article with input from all Authors.

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