The blood genome-wide DNA methylation analysis reveals novel epigenetic changes in human heart failure

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Abstract. – **OBJECTIVE:** Epigenetic mechanisms, such as microRNA, histone modification, and DNA methylation, are critical for dysregulated gene expression in heart failure (HF). However, the relationship between DNA methylation and dysregulated gene expression of blood leukocytes during HF remains unclear.

PATIENTS AND METHODS: In this study, DNA methylation status and gene expression in blood leukocytes from ischemic end-stage cardiomyopathy patients were compared to normal controls by using reduced representation bisulfite sequencing (RRBS), and the results were validated by quantitative MassARRAY analysis and RT-qPCR.

RESULTS: Three differentially methylated genes between two groups were identified. Furthermore, the differential expression of each corresponding gene was found to be correlated with differential DNA methylation. Diverse blood leukocyte DNA methylations existed in HF patients, which were correlated with differential expression of corresponding genes.

CONCLUSIONS: Therefore, detecting DNA methylation in blood leukocytes could be an attractive approach for HF study.

Key Words:
DNA methylation, Epigenetic changes, Blood leukocyte, Heart failure.

Introduction

Heart failure (HF) is a major disease which seriously influences human health, and its morbidity and mortality increase year by year[1,2]. There are annually 870, 000 new HF cases in USA costing almost $30.7 billion in 2012. Although the overall 1-year HF mortality declined owing to the improvements in medical care, heart failure remained at a high level (29.6%)[3].

Multiple molecular and cellular mechanisms are considered to participate in the development and progression of HF. And it shows that the epigenetic regulation plays a pivotal role in the phenotypic response of a failing heart, which takes part in the pathogenesis of HF[4]. Understanding epigenetics in the process of HF may pave the way for early diagnosis, effective therapies, reduced costs and improved life quality for patients. DNA methylation is the most common epigenetic modification in the mammalian genome, and it may play a causative role in HF[5-7]. A recent study showed that global genomic hypomethylation was a risk factor for ischemic heart disease and high total mortality[8], which implies that non-cardiac tissues, such as blood leukocytes that easy to be collected in HF patients, could be used to study the correlation between DNA methylation and HF in clinics.

Comparing to gene chips which employ oligonucleotide hybridization probes targeting CpG sites of interest, whole-genome bisulfite sequencing (WGBS) is more accurate. However, the high cost limited its application in research and practice. Reduced representation bisulfite sequencing (RRBS) is a preferable quantitative assay for investigating DNA methylation at single nucleotide resolution with higher efficiency and lower cost, which could be an alternative approach to WGBS. RRBS can reveal unusual methylated loci and minimizes the DNA loss due to bisulfite-induced degradation, which is especially valuable when sample quantity is limited[9].

We suggested that leukocyte DNA methylation profiles might differ between HF and normal people, which could reflect the differentially regulated gene expression that resulted in HF. To support our hypothesis, we screened and validated the genomic loci with differential methylations through the use of RRBS and quantitative Mas-

sARRAY analysis, and examined its correlation for the gene expression by using RT-qPCR.

Patients and Methods

Ethics Statement
This study was approved by the Medical Ethics Committee at China-Japan Union Hospital of Jilin University. All individuals in the study are provided with written informed consent for the collection of samples and subsequent analysis. This study was conducted according to the principles and guidelines expressed in the Declaration of Helsinki.

Patients
27 individuals with HF and 20 sex- and age-matched healthy controls were enrolled in the study, HF subjects were all from patients admitted to the cardiology clinics at China-Japan Union Hospital of Jilin University with cardiac failure symptoms, and diagnosed with heart function in NYHA IV from 14 Dec 2013 to 30 Apr 2014. The control group was formed by sex- and age- matched 20 healthy volunteers with EF $\geq$ 50% (Table I).

Blood Samples
6 ml blood was drawn into EDTA tubes by venipuncture at the antecubital vein from the patients and controls. The plasma and the blood leukocytes were isolated by gradient centrifugation at 4°C within the first 2 h after collection and stored at -80°C until analysis.

Epigenetic Analysis
Genomic DNA was isolated from white blood cell according to the instruction of the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). RNA was isolated from blood leukocytes using the SV Total RNA Isolation System (Promega, Madison, WI, USA). DNA and RNA concentrations were determined by a NANODROP 1000 (NanoDrop products, Wilmington, DE, USA). Isolated DNA and RNA were stored at -80°C until use.

Reduced Representation Bisulfite Sequencing (RRBS)
For the RRBS studies, 2 pools of DNA were used (each containing 6 subjects); one for control and one for HF. Briefly, 1 μg genomic DNA was digested with the methylation insensitive re-

striction enzyme MspI (New England Biolabs, Ipswich, MA, USA), followed by end-repair and addition of 3' A overhangs. PE or PE index Adapters with a 3' T overhang were ligated to the A-tailed DNA fragments. Fragments between 100 bp and 400 bp were purified by agarose gel extraction (Beijing RuiDaHengHui Science & Technology Development Co., Ltd, Beijing, China). The purified fragments were treated with sodium bisulfite (Tianjin Zhentai Chemical Co., Ltd, Tianjin, China) and then amplified by PCR. The quality of the final PCR product was analyzed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and the ABI SteponePlus Real-Time (Applied Biosystems, Foster City, CA, USA). The final PCR products were then sequenced on the Illumina Hiseq2000 sequencing instrument (Illumina, San Diego, CA, USA). All the works were done by BGI Inc (China).

Quantitative MassARRAY Analysis of Gene Methylation Status
Three samples were chosen from each group to do the quantitative MassARRAY analysis. Primers for genes were designed using the Epi-Designer tool (Sequenom Inc., San Diego, CA, USA) (Table II). $NaHSO_3$ was used to treat the samples before PCR. The PCR cycling profile was: 94°C for 4 min for initial denaturation; 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 45 s, and extension at 72°C for 1 min; and at 72°C for 5 min for a final extension, finally storing at 4°C. After shrimp alkaline phosphatase (SAP) (Qcbio Science & Technologies Co., Ltd, Shanghai, China) treatment, RNase A cleavage, and Resin cleaning, PCR products were analyzed with the Sequenom MassARRAY platform (BGI Inc, China) to quantitatively examine the methylation status of the 3 genes. All the works were done by BGI Inc (China).

Quantitative Real-time RT-PCR (Real-Time RT-qPCR)
Brain natriuretic peptide (BNP) has a cardioprotective effect, and the level of circulating plasma BNP often increases in HF. Before quantifying target gene expression, BNP levels in blood samples were examined, and only the HF samples with at least 6 fold upregulated levels of BNP compared to controls (p=0.046) were selected for analysis. 21 HF samples and 14 Control samples were analyzed by RT-qPCR.

20 μl of cDNA was synthesized from 1 μg of Total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was performed with the cDNA using the SYBR RT-PCR kit on an ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The Real-time PCR assay was performed at 95°C for 10min, 40 cycles of 95°C for 15 s, finally followed by 60°C for 1 min. Primers for the RT-qPCR were synthesized by

Table I. This table shows the characteristics of the different samples. 1-6 and A-F were for RRBS; 7-9 and G-I were for MassARRAY; 7-27 and G-T were for RT-PCR; 7-16 and G-N were for ELISA.

Code	Sex	Age	Details	Heart function	EF (%)
1	F	76y	OMI, HTN, DM	NYHA IV	21
2	M	69y	OMI, DM	NYHA IV	38
3	M	59y	Ischemic cardiomyopathy, DM	NYHA IV	35
4	F	58y	Ischemic cardiomyopathy	NYHA IV	32
5	F	63y	Ischemic cardiomyopathy, DM	NYHA IV	24
6	M	71y	Ischemic cardiomyopathy	NYHA IV	29
7	M	72y	Ischemic cardiomyopathy	NYHA IV	26.5
8	F	62y	OMI	NYHA IV	39
9	F	79y	Ischemic cardiomyopathy	NYHA IV	27
10	M	70y	Ischemic cardiomyopathy, DM	NYHA IV	42
11	M	87y	Ischemic cardiomyopathy	NYHA IV	41.5
12	M	64y	Ischemic cardiomyopathy, HTN	NYHA IV	39.5
13	F	69y	Ischemic cardiomyopathy, AF, DM	NYHA IV	47
14	F	51y	OMI, DM, HTN	NYHA IV	48
15	M	60y	Dilated cardiomyopathy, HTN	NYHA IV	34
16	F	64y	OMI, HTN	NYHA IV	35
17	F	70y	Ischemic cardiomyopathy	NYHA IV	43
18	F	67y	Ischemic cardiomyopathy, DM	NYHA IV	45
19	M	55y	OMI, DM	NYHA IV	40
20	F	76y	Ischemic cardiomyopathy	NYHA IV	50
21	F	79y	Ischemic cardiomyopathy, HTN, renal failure	NYHA IV	48
22	M	66y	Ischemic cardiomyopathy, HTN, renal failure	NYHA IV	48.5
23	F	70y	Ischemic cardiomyopathy, AF, HTN	NYHA IV	42
24	F	95y	Ischemic cardiomyopathy, AF, HTN	NYHA IV	38
25	F	68y	Ischemic cardiomyopathy, AF	NYHA IV	48.5
26	M	78y	Ischemic cardiomyopathy, HTN, DM	NYHA IV	46.5
27	M	42y	Ischemic cardiomyopathy, HTN	NYHA IV	49
A	F	52y			60
B	M	60y			65
C	M	65y			57
D	F	64y			57
E	M	57y			60
F	F	61y			58
G	F	69y			55
H	F	68y			54
I	M	54y			60
J	M	61y			53
K	M	54y			60.5
L	M	51y			57
M	F	73y			52
N	F	54y			63
O	F	65y			63
P	F	45y			61
Q	M	39y			59
R	F	53y			58
S	M	48y			70
T	F	40y			71

Note: OMI: old myocardial infarction; DM: diabetes mellitus; HTN: hypertension; AF: atrial fibrillation.

Table II. Sequence of primers for MassARRAY.

Gene	Primer	Sequence (5′ to 3′)
MPV17L	5' Primer	aggaagagagTGTGTTAATGGATTTATGTATGGGG
	3' Primer	cagtaatacgactcactatagggagaaggctCCAACCTCCAACAACTACTACACTA
PLEC	5' Primer	aggaagagagTAGTTTTGATTTGTTAGGTTGGAGG
	3' Primer	cagtaatacgactcactatagggagaaggctACCCTATTCCAAACTCTACAACAACT
SLC2A1	5' Primer	aggaagagagGAGGTTGAGGTAGAGAATTGTTTGA
	3' Primer	cagtaatacgactcactatagggagaaggctCAAAAATTAACTTCTCCCTTCCCTA

Sangon Biotech (Shanghai, China) (Table III). For the data analysis, the comparative cycle threshold (C_T) value for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the loading variations in the RT-PCR reactions. The ΔC_T value was then obtained by subtracting the control $\Delta\Delta C_T$ values from the corresponding experimental $\Delta\Delta C_T$ values. The $\Delta\Delta C_T$ value was converted into a fold difference that was compared with the control using the equation $2^{-\Delta\Delta CT}$.

Enzyme-linked Immunosorbent Assay (ELISA)

10 HF samples and 8 control samples were analyzed to examine the relevant gene expression in blood leukocytes by ELISA. The total protein from the leukocytes was extracted by protein pyrolysis buffer. The expression level of protein was determined with a human ELISA test kit (IBL, GmbH, Hamburg, Germany) according to the manufacturer's instructions.

Statistical Analysis

SPSS 19.0 (Version 19.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data for RT-qPCR, quantitative MassARRAY and ELISA was analyzed by the non-parametric Mann-Whitney *t*-test, and two-tailed *p*-values were used to determine statistical significance. Analysis for the association between DNA methylation and gene expression was performed using Spearman's rank order correlation coefficient. All reported *p*-values were two-tailed, and statistical significance was set at *p*<0.05.

Results

Detailed Characteristics of Patients and Control Subjects

The detailed characteristics of patients and control subjects were shown in Table I. There were no significant age and gender differences between patient and control groups. For RRBS, blood leukocytes from 1-6 (patients) and A-F (controls) were used. For quantitative MassARRAY methylation analysis, blood leukocytes from 7-9 (patients) and G-I (controls) were chosen. Blood leukocytes from 7-27 (patients) and G-T (controls) were used for RT-qPCR. Blood leukocytes from 7-16 (patients) and G-N (controls) were used for ELISA analysis.

Reduced Representation Bisulfite Sequencing (RRBS)

To determine DNA methylation in peripheral blood, we profiled one genomic DNA pool composed of 6 peripheral blood samples from HF patients as well as one genomic DNA pool composed of 6 peripheral blood samples from non-HF subjects as a control. We generated 120 million clean reads for HF pool and 114 million clean reads for control pool. Of these, 71.99% was uniquely mapped to either strand of the human genome (hg19) in HF samples and 71.82% in normal samples. We were able to determine the methylation status of approximately 10,680 CpG sites (CPGs) in HF pool and 11,228 CPGs in normal one consistently. The overall distribution of CpGs, as well as methylated CpGs in the repetitive sequences was also consistent between samples. These CpGs account for more than 43% of annotated CpGs in the genome examined, and

Table III. Sequence of primers for RT-PCR.

Gene	Primer	Sequence (5′ to 3′)
MPV17L	5' Primer	CTCGGCCTTCTATGTCGGTA
	3' Primer	GGGCCAGTACATCAGTCCAC
PLEC	5' Primer	GCTTCAGTGGATGCGACAC
	3' Primer	GATTGGTAGATGCCCTTGGA
SLC2A1	5' Primer	AGGGTAGGAGGTTTGGATGG
	3' Primer	TGAGCAAGAGGACACTGATGA

48%-56% of CpGs analyzed were located in the 5' end regulatory and coding sequences. Analysis of average DNA methylation in regions annotated as genes showed a bimodal distribution with enrichment of highly methylated peaking around 90-100% methylation and less methylated sequences peaking around 10-30% methylation (Figure 1). And we found that whether promoter or gene body, the heart failure's had a decrease DNA methylation level than the normal ones.

Identification of Differentially Methylated Regions (DMRs)

To identify the DMRs between HF samples and normal samples, we performed a genome-wide unbiased DMR detection by determining the differences in the CG site methylation in the same genomic region of the two groups. In total, we identified 732 DMRs that were hyper- or hypo-methylated in HF pool compared with Non-HF pool. And 21.2% of the DMRs were located in the promoter regions, while 78.8% of the DMRs were located in the intergenic or intragenic regions. The hypermethylation of gene promoters in heart failure is related with metabolism pathway such as beta-Alanine metabolism, phenylalanine metabolism and their function focus on regulation of transcription, oxygen species metabolic process, apoptotic process. And the hypomethylation of gene promoters in heart failure is related with complement pathway and their function focus on cell proliferation, regulation of apoptotic, complement activation. In addition, in heart failure, the hypermethylation in gene body of genes is related with tight junction pathway, and their function focus on transcription, calcium ion binding, regulation of GTPase activity, and the hypomethylation in gene body of genes is related with adherens junction, hippo signaling pathway, and their function focus on regulation of transcription.

The functional annotation analysis generated using DAVID showed that about 22.46% of these proteins were the components of plasma membrane and 18.25% of these proteins regulate transcrip-
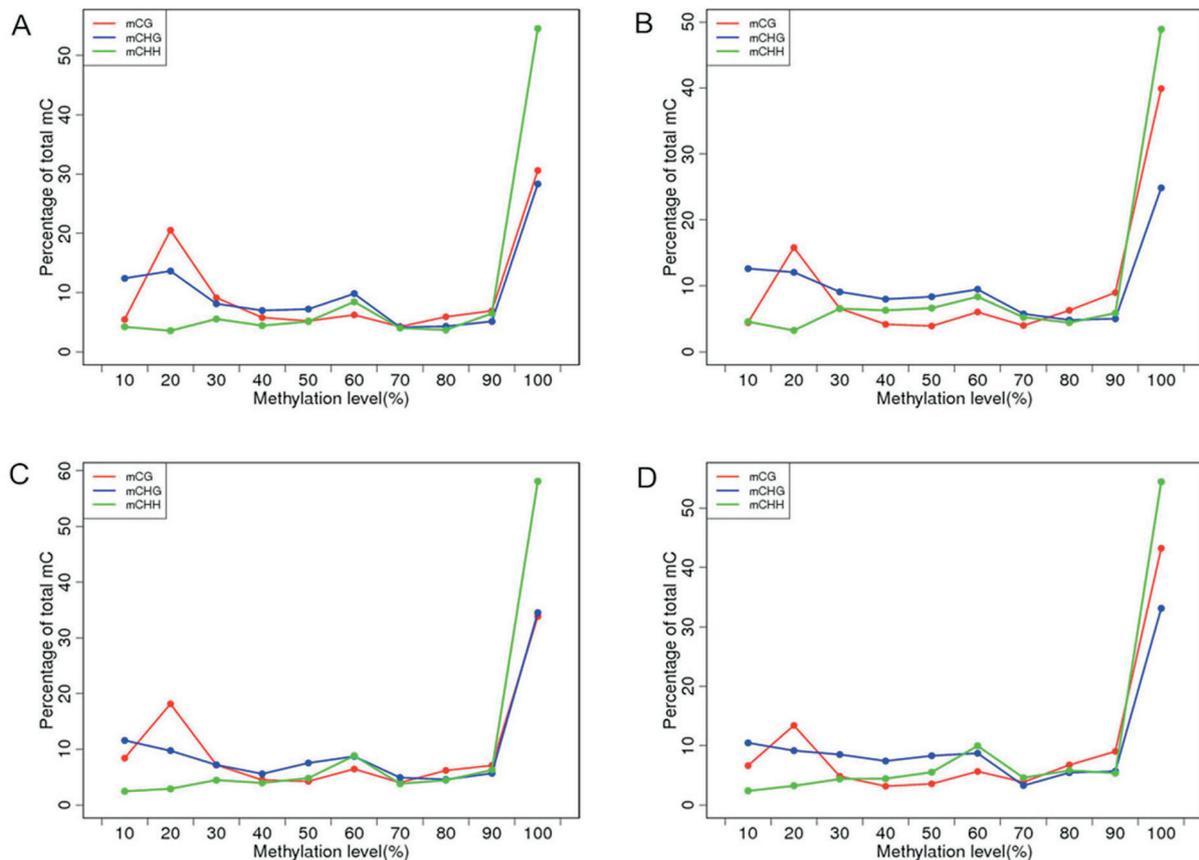

Figure 1. Average DNA methylation in regions annotated as CGI and promoter of two groups. Line graphs of average DNA methylation calculated for *(A)* CGI of control, *(B)* promoter of control, *(C)* CGI of heart failure, *(D)* promoter of heart failure.
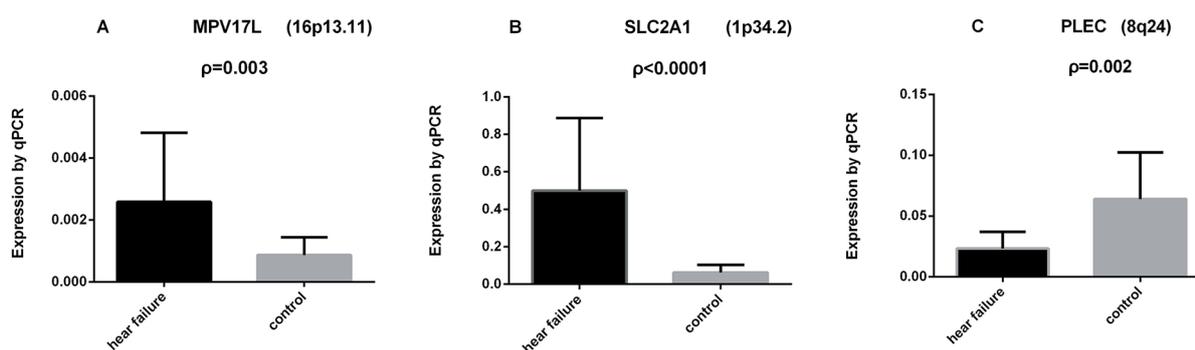
Figure 2. Results of quantitative RT-PCR analysis for 3 genes (SLC2A1, PLEC, MPV17L) between two groups. RT-PCR was performed in a set of 35 blood samples (14 controls: G-T, 21 HF: 7-27). RT-PCR experiments were performed in triplicate for each sample.

tion. Based on the results from Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis, the 732 genes are enriched in several pathways (i.e. Adherens junction p-value=0.00889, pathways in cancer p-value=0.02228, maturity onset diabetes of the young p-value=0.0397, prostate cancer p-value=0.054).

Three candidate genes were identified and selected by gene ontology analysis and GENECARDS. We chose 3 genes participated in the cellular component, related with metabolism and junction. (1) SLC2A1 gene, encoding a glucose transporter protein; (2) MPV17L gene, encoding a protein participating in cellular apoptosis; (3) PLEC gene, encoding a protein involved in actin binding and ankyrin binding. All the three genes can play roles in HF.

Results by Quantitative MassARRAY Analysis

To determine the DNA methylation level of the three candidate genes, we performed quantitative MassARRAY to analyze DMRs in 3 HF samples and 3 non-HF samples. Bisulfite treatment of gDNA converts unmethylated-cytosine nucleotides to uracil, but leaves methylated-cytosine residues unaffected. After restriction digestion, the size and molecular weight of DNA depend on the nucleotide variation after treated by $NaHSO_3$, which can be examined by quantitative MassARRAY. Quantitative MassARRAY has high sensitivity and can check at the methylation level lower than 5%.

The DNA methylation levels of MPV17L were 5.7%, 6.7%, and 6.5% respectively in 3 HF samples, while the DNA methylation levels in 3 control samples were 8.2%, 7.8%, and 8.8% respectively. The DNA methylation levels of SLC2A1 were 21.4%, 37.9%, and 14.3% respectively in 3 HF samples, while the DNA methylation levels in

3 control samples were 37.4%, 41.9%, and 34.7% respectively. The DNA methylation levels of PLEC were 63.8%, 83.3%, and 62% respectively in 3 HF samples, while the DNA methylation levels in 3 control samples were 59.1%, 60.1%, and 57% respectively. The DNA methylation level of MPV17L and SLC2A1 were lower in HF group than control, while the level of PLEC was higher in HF group than control. But there is no significant difference between 2 groups, just a trend.

Results by Quantitative RT-PCR Analysis

Three genes (SLC2A1, MPV17L, PLEC) with DMRs between the two groups were selected for further analysis by quantitative RT-PCR.

Data from RT-qPCR analysis showed that in HF samples MPV17L and SLC2A1 had an increasing gene expression in HF, while PLEC had a decreasing gene expression. And there were statistic differences in target mRNA expression between HF and control leukocytes (Figure 2, $p < 0.001$ for SLC2A1, $p = 0.003$ for MPV17L, and $p = 0.002$ for PLEC).

Correlation Between Differential DNA Methylation and Gene Expression

We used the data from quantitative MassARRAY analysis and RT-qPCR to identify the relationship between differential DNA methylation and gene expression. As predicted, we found that 5' region methylation correlated inversely with gene expression (Figure 3). Contrary to reported effect of methylation in gene body, hypermethylation in PLEC gene body correlated with reduced mRNA expression.

The Quantification of Gene Related Proteins

Data from ELISA analysis showed that the SLC2A1 and MPV17L protein levels were higher in

leukocyte of HF patients compared with controls, and the PLEC protein level was lower in leukocyte of HF patient compared with the control, which was consistent with the results of RT-qPCR. The protein concentrations of SLC2A1, MPV17L and PLEC in leukocytes were 5286.3±2558.2 pg/ml (p=0.007), 376.1±287.8 pg/ml (p=0.04) and 35970.4±17646.3 pg/ml (p=0.03) respectively in HF patients, while the protein levels in controls were 2478.9±614.6 pg/ml, 155.3±64.9 pg/ml and 86773.4±47142.4 pg/ml respectively.

Discussion

In the present work, we investigated the DNA methylation patterns of blood leukocyte on a genome-wide level in HF patients, confirming some significant aberrant DNA methylations in a number of CGIs. This suggested that DNA methylation alterations in blood leukocytes were associated with HF.

In eukaryotic cells, gene expression is regulated by genetic and epigenetic mechanisms. Epigenetics are recognized as a key to understand of pathogenesis CV diseases, which influences gene expression without altering the DNA sequence and can be transmitted between cell generations[10,11].

DNA methylation is an important epigenetic modification occurring almost exclusively in the context of CpG dinucleotides[12]. Recently, the investigations in DNA methylation of HF were done in left ventricular tissue, and they found some genes' methylation play a causative role in the programming of heart hypertrophy and reduced global cardiac contractility function, then lead to HF[13-16]. While our research firstly demonstrates that differential DNA methylation existed in HF

patients' blood leukocytes comparing to normal controls. Myocardium is believed to be a better tissue to investigate the epigenetic mechanisms of HF. However, it is always challenging to collect myocardium samples in clinics, especially for normal controls. In contrast, blood samples for both HF and normal controls are easy to get. It should be noted that only six samples were included in the DNA pools for each group. Therefore, we used quantitative MassARRAY methylation analysis to confirm the consistency of the two analysis dataset in DNA methylation, and more samples were used to perform analysis of gene expression by RT-qPCR to verify the results of RRBS and quantitative MassARRAY methylation analysis. Our data confirmed that differential DNA methylation was related to differential gene expression in three candidate genes.

We analyzed the methylation status of approximate 10,680 CPGs in HF patients' blood leukocytes and 11,228 CPGs in normal controls' blood leukocytes, which led to the discovery of 732 DMRs. Since one chr X in female was inactivated and our sequencing was done in both female and male, the DMRs in chr X was not analyzed in the present study. And we found that there is a decrease DNA methylation in genomes of HF patients, which is consistent with the previous study. It reminds that hypomethylation is a risk factor to HF, which playing a significant role in the process of HF[6]. As shown in our results, methylation level of DMRs in promoter regions had a negative correlation with gene expression levels, which is consistent with previous studies. However, a negative correlation was observed in PLEC gene between gene expression and methylation level of DMRs in gene body, which seems to be contrary to the common believes that higher DNA methylation
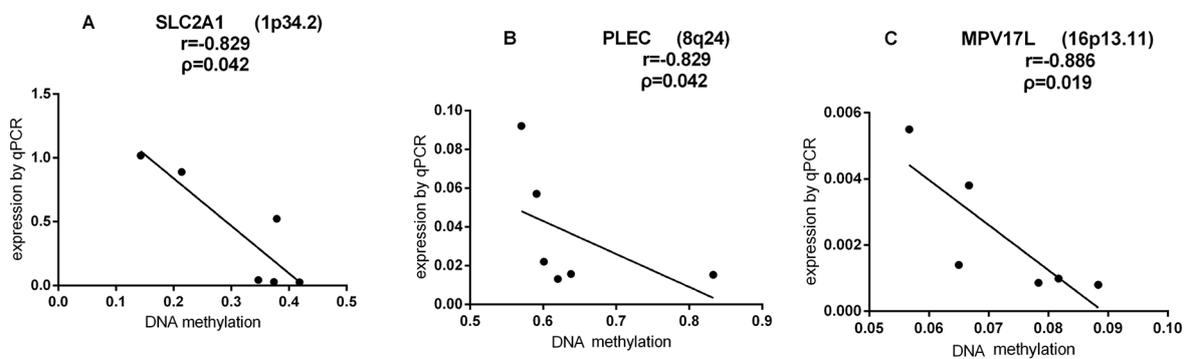

Figure 3. Differential DNA methylation for 3 DMRs correlates with differential gene expression. (A) SLC2A1, (B) PLEC, (C) MPV17L. Correlation between gene expression and DNA methylation using Spearmans rank order correlation coefficient.

level in gene body is associated with increased gene expression. It is possible that these methylation sites in PLEC gene body represented functional elements, such as enhancers or alternative promoters under normal circumstance[17].

Plectin, a multifunctional cytolinker protein from PLEC gene, is considered as a universal cross-linking element of the cytoskeleton[18]. Plectin is expressed at intercalated disks of cardiac muscle, dense plaques of smooth muscle, Z-lines of striated muscle[19]. Previous researches[20-22] have shown that dysfunction of plectin could lead to cardiomyopathy due to the disintegration of intercalated disks in the heart. In HF patients, the lower expression level of plectin was found in blood leukocytes caused by DNA methylation, which could aggravate the condition of HF.

MPV17L (MPV17 mitochondrial membrane protein-like) protein is a member of the MPV17/PMP22 transmembrane protein family[23]. MPV17L protein is a negative regulator of apoptosis and hydrogen peroxide biosynthetic process through binding with HtrA2 to form a unique mitochondrial protein complex[24]. In HF patients, higher expression of MPV17L was showed in blood leukocytes due to DNA methylation. This elevated expression of MPV17L could be part of the protective mechanism during HF.

Glucose transporter 1 (GLUT1) is an insulin-independent glucose transporter, which is widely expressed as a mediator of basal cardiac glucose uptake in quiescent myocytes. Despite the developmental downregulation of GLUT1 soon after birth, the expression of GLUT1 would increase in hearts under several pathophysiological states, such as pressure-overload hypertrophy[25,26]. It is reported that increased expression of GLU1 isoform was associated with the hypertrophy of rat neonatal ventricular myocytes[27]. Liao et al[28] demonstrated that GLUT1 could prevent the development of HF and improve the survival of mice. Furthermore, Morissette et al[26] found that up-regulation of GLUT1 is necessary for hypertrophy, implying that it serves as a signaling molecule during cardiac hypertrophy. Increased expression of GLUT1 mediates increased survival of myocytes. However, no changes were observed in glucose uptake at overexpressed or inhibited expression of GLUT1. Hence, alternative mechanisms might exist in mediating the myocyte hypertrophy and survival through the GLUT1. In HF patients, we found that there was a higher expression of GLUT1 in blood leukocytes, which is caused by DNA methylation.

Conclusions

We showed that differential gene methylation occurs in blood leukocytes of HF patients compared with normal controls. Compared with existing methods, our approach is an easier alternative in using leukocyte to study the association between DNA methylation and HF. Some of the genes in this study might potentially serve as new HF biomarkers for early detection or even as therapeutic targets. However, due to the small sample size, studies with large sample size are needed to further validate the results from this study.

Ethics Approval and Consent to Participate

The medical Ethics Committee at China-Japan Union Hospital of Jilin University approved the study. All the patients give their informed consent.

Conflict of interest

The authors declare no conflicts of interest.

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