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Effect of Metformin on the Epigenetic Age of Peripheral Blood in Patients With Diabetes Mellitus (DM)

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Research Article

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Abstract

Background: Metformin has been proved to have anti-aging effect. However, studies on how metformin affects global epigenetic regulation and its effect on the epigenetic clock in the diabetes mellitus (DM) patients is limited. This study aims to investigate the impact of metformin on the epigenetic age in subjects with type 2 DM.

Methods: We collected the peripheral blood of metformin group and the no metformin group of the 32 DM patients. Epigenetic clock was used to estimate the epigenetic age acceleration of the two groups.

Results: The results were presented as the following: 1) the metformin slow down the epigenetic age of the DM patients by an average of 2.77 years. 2) we observed a significant enrichment in the regulation of histone methyl-transferase activity (H3K9 specific) 3) we identified 144 differentially methylated positions (DMPs), the top 20 cytosine–guanine dinucleotides (CpGs) and their associate genes with the most consistent changes in the DNA methylation profile were selected: EPM2AIP1, MUC17, FNDC1, HLA-DOB, TRPC4, ESRRB, ADCY2. The major functions of most top-level differential methylation sites and the cellular signaling pathways they represent are related to aging process.

Conclusions: Here we demonstrated that metformin could perform an anti-aging effect by slow down the epigenetic clock.

Background

DNA methylation is the mostly widely studied epigenetic mark, it is the process by which methyl groups are added or removed from the DNA sequence, usually at cytosine–guanine dinucleotides (CpGs). DNA methylation patterns change over the life in response to environment factors such as diet, smoking, stress and it also changes with age [1] [2] [3]. For example, while hypo methylation is common with aging, some CpG islands and gene-rich regions become hyper methylated with age [4]. Based on age-related changes in DNA methylation, several research groups have identified what are known as DNA methylation clocks [5] [6] [7]. The epigenetic clocks has been established to be predictive of all-cause mortality [8] [9], cancer [10] [11], frailty, and cognitive and physical functioning [12]. These DNA methylation clocks have been identified to predict chronological age with high accuracy and considered as the most promising marker of aging [13]. Age acceleration, discrepancy between DNAm age and chronological age, tells us whether the person is biologically younger or older compared to his/her chronological age [14] [15]. The accelerated epigenetic aging has been proved to be associated with many aging related and other disease, including cancer, Down's syndrome, physical and cognitive decline, all-cause mortality [12] [16] [8] [17].

Metformin is a widely used medication that has been used as the first line oral treatment for type 2 diabetes [18]. Recent advances revealed that this drug, in addition to its glucose-lowering action, might be promising target for aging [19] [20] [21] [22] [23]. It appears to target a number of age-related mechanisms. At a molecular level, metformin leads to activation of adenosine activated protein kinase

(AMPK) and increases antioxidant protection [20] [21]. Metformin could exert the inhibition of mammalian target of rapamycin (mTOR) signaling [24]. Inhibition of this pathway extends lifespan in model organisms and confers protection against a growing list of age related pathologies [22]. Preclinical studies of metformin suggest Metformin robustly increases lifespan in C. elegans by up to 36% [23]. There is a lot of evidence that metformin is a promising anti-aging drug. However, studies on how metformin affects global epigenetic regulation and its effect on the epigenetic clock is limited. The aim of the study was to investigate the metformin induced anti-aging effect and its effects on the genome-wide DNA methylation in human peripheral blood. We conducted this study to investigate the pathways of metformin in real-life physiological conditions in humans. This is important given the polypotent effects of metformin, and such research could lead to new and important targets not only for the treatment of DM, but also for other diseases.

Methods

2.1 Study population

Diabetes mellitus (DM) was defined as the presence of diabetes symptoms and a resting plasma glucose concentration ≥ 200 mg/dL, a fasting plasma concentration ≥ 126 mg/dL, a 2-h plasma glucose concentration ≥ 200 mg/dL in a 75 g oral glucose tolerance test, or use of a hypoglycemic agent or other medications for DM. The candidates were excluded if they had severe heart failure, active infectious disease, history of malignancy, or end stage of renal disease, or were in a deep coma. Patients were also excluded if they have any other chronic disease other than diabetes, such as cardiovascular disease, respiratory disease, tumor, rheumatic immune disease, confirm that the participants did not use antibiotics, immunosuppressive medications, corticosteroids, or pharmaceutical-grade probiotics during at least two months leading up to blood collection. Also confirm that no subject had diarrhea within 7 days leading into the study. All of the participants reported that they were on a normal diabetic diet. These patients were divided into two groups based on whether they were on metformin medication with a stable dosage of 0.5 g/d for at least of 5 years. The characteristics of the study population were summarized in Table 1. None of the two groups included in the study had any disease other than diabetes and neither group had received any other medications in the recent five years. All participants provided written informed consent and the study was approved by the ethics board of Chinese PLA General Hospital.

2.2 Genomic DNA extraction and quality control

Fasting peripheral blood samples were collected in the morning and then stored at - 80°C until use. Approximately, 500 ng of genomic DNA from each sample was used for sodium bisulfite conversion using the EZ DNA methylation Gold Kit (Zymo Research, USA) in accordance with the manufacturer’s instructions. DNA from a total of 32 participants were assayed using Illumina’s Infinium Human Methylation 850 Beadchip as previously described. Genome-wide DNA methylation was assessed using the Illumina Infinium Human Methylation 850K BeadChip (Illumina Inc, USA) which covers 99% of all

RefSeq genes and contains 853,307 sites. The process was performed according to the manufactures protocols. Quality control of methylation data including intensity read outs, filtering, cellar composition, was adapted from Hannon et al and was done using the R package (version 4.0.0) [25]. Load EPIC's original IDAT files and filter out probe sites according to the following principles: a) Filter out probes used to filter P values (≧ 0.01) b) In more than 5% of samples the probes in beads smaller than three were filtered out c) Filter out all non-CpG probes contained in the data set d) Filter all SNP-related probes e) Filter all probes in chromosome X and Y.

2.3 Determinations of epigenetic age and epigenetic age acceleration

The array data (IDAT files) was analyzed using ChAMP package in R for deriving the methylation level. The methylation status of all the probes was denoted as β value, which is the ratio of the methylated probe intensity to the overall probe intensity (sum of methylated and unmethylated probe intensities plus constant α, where α = 100). CpG site (probe) intensities were transformed to β values with a standard equation in which beta is the ratio of the methylated probe (m) intensities to the overall intensities (m + u + α, where α is the constant offset, 100, and u is the un-methylated probe intensity). The resulting β values ranged from 0 (completely un-methylated) to 1 (fully methylated). It is generally believed that a β value greater than 0.8 is Hypermethylated, while a β value less than 0.2 is Hypomethylated, and a β value in the range of 0.2 ~ 0.8 is partially methylated. The DNA methylation ages were measured using the epigenetic clock [5]. The epigenetic clock was defined as an age prediction method based on DNA methylation levels at 353 CpG sites. The Acceleration Diff value was calculated by subtracting the actual chronical age from the DNAm age [5].Another measure of acceleration (Acceleration Residual) equaled the residual resulting from linear regressing DNAm age on chronological age [5]. Epigenetic clock procedures were performed based on the instructions on the website (https://dnamage. genetics.ucla.edu).

2.4 Differentially methylated positions between the two groups.

CpG sites having $|\Delta\beta|\geq 0.20$ (in test vs control) and adjusted P value ≤ 0.05 was considered as differentially methylated site. A CpG was considered hypermethylated if $\Delta\beta \geq 0.20$ or hypomethylated if $\Delta\beta \leq -0.20$. Average β value of promoters and CpG islands were compared between disease and normal. Promoters and CGIs with $|\Delta\beta| \geq 0.20$ and adjusted P value ≤ 0.05 were considered for further analysis.

2.5 GO function analysis

The functions of genes was assessed by GO analysis and pathway network analysis. The methylated sites was mapped to GO terms in the Gene Ontology database. GO analysis is divided into three parts: molecular function, biological process, and cellular component.

2.6 Statistical analysis

Baseline characteristics was conducted by Stata 14 software (StataCorp, Inc., College Station, TX, USA). The data are presented as averages and standard deviations unless otherwise stated. Associations between chronological age and DNAm age were analyzed using standardized regression coefficients. The Kruskal-Wallis test was performed to determine the significant difference between the metformin group and the control group. R Studio was used to perform the statistical analysis. R package was used to implement the meta-analysis. The forest plot was drawn by the forest plot. R package. We used Pearson correlation analysis to verify whether the mythlation of the DMPs mapped to different genes were associated with gene expression.

Results

3.1 Characteristics of the study participants

All subjects were male, with an average of 73.3 years. Based on the medications of metformin, the patients were divided into no-metformin group and metformin group. 16 DM patients with at least of 5-year medication with a stable dosage of 0.5 g/d were included in the metformin group and the other 16 patients with no metformin medications in the recent 5 years were included in the no-metformin medication group. A total of 32 patients was included in the study. The characteristics of the study population were summarized in Table 1.

Table 1
Characteristics of the study group

Characteristics of the study group				
Characteristics	Total	No-metformin group	Metformin group	P value
Male	100%	100%	100%	-
Age, years, mean ± SD	73.3 ± 5.60	73.6 ± 5.90	72.9 ± 6.30	0.56
Smoking, n (%)	12(37.50)	5(31.25)	7(43.75)	0.09
BMI, mean ± SD	25.3 ± 3.40	25.2 ± 2.90	25.4 ± 3.70	0.98
Fasting plasma glucose, mmol/l, mean ± SD	6.27 ± 2.70	6.19 ± 2.08	6.31 ± 3.12	0.12
HbA1c,%	(5.35 ± 1.07)%	(5.65 ± 0.98)%	(5.24 ± 1.20)%	0.65

3.2 Prediction of epigenetic age using the epigenetic clock

We used Illumina Infinium 850 k array to evaluate the effect of metformin on DNA methylation in patients. The mean age was 73.6 for metformin group and 73.3 for no metformin group (p = 0.56). To verify the epigenetic clock predictors in our cohort, we correlated epigenetic age with chronical age, as described previously. We found that the DNAm age had a strong linear relationship with the chronological

age ($r = 0.85$, 95%CI: 0.71–0.92, $P < 0.0001$) (Fig. 1a). The correlation was seen both the metformin group ($r = 0.89$, 95%CI: 0.72–0.96, $P < 0.0001$) (Fig. 1b) and no metformin group ($r = 0.86$, 95%CI: 0.64–0.95, $P < 0.0001$) (Fig. 1c).

3.3 Metformin slow down aging of peripheral blood in patients with DM

T tests was conducted to verify the whether metformin slow down aging in the peripheral blood of the patients with DM (Fig. 2). We evaluate age acceleration developed by Horvath in two ways. The first is the Acceleration Diff, which is equal to the DNAm age minus the chronological age; the second is Acceleration Residual, based on linear regression residue of methylation age and chronological age [5]. A positive (negative) value of accelerated age indicates that the DNAm age is older (younger) than its actual age. The Acceleration Diff value of no metformin group exceeded that of metformin group by 2.77 years ($P = 0.04$) (Fig. 2a). The Acceleration Residual values did not show significant differences between the two groups, but these differences show a similar trend to accelerating differences (Fig. 2b).

3.4 Differentially methylated CpGs

After the homogenized β values are obtained, the CHAMP package is used for the differential methylation positions (DMP) analysis between groups. Compared with the non-metformin group, 144 positions of 853,307 positions were differentially methylated (Benjamini-Hochberg (BH)-adjusted p value < 0.05) (Fig. 3). Volcano plots showing the different DNA methylation positions (Fig. 4). As Fig. 4 shows, the DMPs were eventually distributed across all autosomes. Of these positions 56 positions were hyper-methylated and 88 positions were hypo-methylated. Among the identified DMPs, a total of 20 CpGs with the most consistent changes in the DNA methylation profile were emphasized. Twenty CpG sites that were also significant after Holm-Bonferroni correction (Table 2). The identified CpG sites respond to 7 genes according to the 850 k annotation file published previously. The seven genes were EPM2AIP1, MUC17, FNDC1, HLA-DOB, TRPC4, ESRRB, ADCY2.

Table 2
Top twenty differentially methylated probes associated with metformin

CpG site	Gene	Location	p value	Δβ
cg27155888	-	IGR-opensea	0.00000617	-0.112749496
cg12472342	-	IGR-opensea	0.0000241	-0.034778095
cg04027059	-	IGR-opensea	0.0000401	0.046771263
cg16516576	-	IGR-opensea	0.0000465	-0.03718775
cg08396483	-	IGR-shore	0.000053	-0.1074229
cg24607398	EPM2AIP1	1stExon-shore	0.0000541	0.029846289
cg23858082	MUC17	3'UTR-opensea	0.0000561	0.036155944
cg09698277	FNDC1	Body-opensea	0.000247197	-0.072351985
cg01548742	-	IGR-shelf	0.000259535	-0.112348121
cg07301677	-	IGR-opensea	0.000272337	-0.139337199
cg05490803	-	IGR-opensea	0.000279813	0.034232439
cg04348707	HLA-DOB	Body-opensea	0.000325737	0.039684197
cg19591490	-	IGR-opensea	0.000346629	0.028808422
cg02124233	-	IGR-opensea	0.000371765	-0.025197641
cg24680646	TRPC4	Body-opensea	0.000373824	-0.12260603
cg00028022	-	IGR-opensea	0.00037748	-0.109780335
cg07730622	ESRRB	5'UTR-opensea	0.000559083	-0.13784472
cg03739434	ADCY2	Body-opensea	0.00063895	0.05576932
cg20757404	-	IGR-shelf	0.000674122	-0.039658001
cg06174194	-	Body-opensea	0.000695738	-0.349479356

3.5 Enrichment analysis

To evaluate the potential biological significance of the effects of different methylated CpG sites, we performed gene-set pathway enrichment analysis. Enrichment analysis of DNA methylation results was performed using a tool provided by the Network Gene Set Analysis Kit (Web-Gestalt). GO analysis were performed. Significant differentially methylated probes with FDR < 0.05 was considered significant. The result is divided into three parts: molecular function, biological process, and cellular component. The histogram of GO with significant enrichment is listed in Fig. 5. GO analysis is divided into three parts: molecular function, biological process, and cellular component. We saw a significant enrichment in the

regulation of synapse assembly in the biological process, actin filament in the cellular component part. Moreover, we observed a significant enrichment in the regulation of histone methyl-transferase activity (H3K9 specific) in the part of molecular function.

Discussion

We performed a genome-wide methylation study to investigate the effect of metformin on DNA methylation using baseline blood samples. We demonstrated that metformin could slow down the epigenetic clock thus inducing anti-aging effect. In the present study, we also identified 144 CpG sites that were differentially methylated between metformin group and the no-metformin group (FDR < 0.05). The differentially methylated sites in our study were similar to those identified in previous studies on anti-aging effect [26–28]. Our study strengthening the view reported previously that epigenetic clock is a relatively accurate reflection of a person's biological age. However, unlike any of the previous studies, studies on how metformin affects global epigenetic regulation and its effect on the epigenetic clock is limited.

4.1 Metformin and aging

Metformin is an approved drug to treat diabetes, but it also seems to target some of the mechanisms associated with aging. Especially for aging, metformin can lead to decreased insulin levels, decreased IGF-1 signaling pathway [29] [30], and mTOR inhibition [31] [32], inhibition of mitochondrial complex 1 in the electron transport chain and reduction of endogenous reactive oxygen species (ROS) production [33] [34], activation of AMPK [32] [35], decrease of DNA damage [36] [37]. It has been reported that the metformin increases lifespan of C. elegans by altering microbial folate and methionine metabolism [23]. It also improves healthspan and lifespan in mice and human [21] [38]. Moreover, metformin has been proved to interact with several known longevity pathways such as dietary restriction (DR) [39] [40].

4.2 The epigenetic clock

Recent evidence suggests that the epigenetic clock is the most promising marker of ageing. The epigenetic clock has been reported to track biological aging and associated with morbidity and mortality. The result of a meta-analysis including 13,089 participants showed that epigenetic clocks can predict all-cause mortality [9]. Epigenetics clock is considered to be the most promising biomarker of biological age when compared with telomere length and other biomarkers [41]. Similarly, by comparing different estimation methods, the researchers identified DNA methylation as the most promising biomarker for predicting age [42]. Therefore, many anti-aging measures use epigenetic age to evaluate the effectiveness of interventions [43] [44]. Metformin has long been considered as an “anti-aging” drug, based on preclinical experiments with lower-order organisms and numerous retrospective data [19]. However, the molecular mechanisms remained unclear and the underlying mechanisms need to be better understood. Previous studies have reported effect of metformin on the epigenetics [45] [46] [47]. In a small sample size study (n = 12), Elbere and colleagues have shown an altered blood DNA methylation profile following metformin use in non-diabetic participants [48]. However studies focused on metformin's effect on the

epigenetic clock is limited. The analysis of epigenetic differences between elderly diabetic patients with and without metformin is helpful to find possible intervention targets.

4.3 The underlying mechanisms: Histone methyltransferases and AMPK.

Our study confirm that epigenetic ages are younger in DM patients with medication of metformin. Further GO function analysis was performed, suggesting that metformin affects the activity of histone methyltransferase (H3K9 specific).

AMPK plays a major regulatory role in cell energy homeostasis by directly phosphorylating metabolic enzymes and nutrient transporters, and indirectly promoting mitochondrial biogenesis and the de-activation of nuclear genes in functional mitochondrial biogenesis. AMPK as a target for promoting healthy aging is associated with its role in multiple signaling pathways. a) TOR pathway: down-regulation of the TOR pathway extends lifespan in C. elegans, fruit flies, and mice [22]. The prevailing view of AMPK/TOR interaction is that AMPK primarily as an upstream inhibitor of TOR [49] [50]. b) FOXOs pathway: rIIS is the most powerful and least controversial candidate to promote healthy aging, rIIS dramatically increases life expectancy and extends healthy aging in a variety of organisms, including mammals. The only member of the FOXO transcription factor family that is activated by rIIS completely requires RIIS-mediated longevity. In C. elegans, AMPK might activate FOXO thus performing the anti-aging effect. c) Sirtuins pathway: SirT1 gene plays an anti-aging role by improving efficiency in inducing and maintaining pluripotent states. AMPK can activate Sirt1 by changing the nicotinamide adenine dinucleotide: reduced nicotinamide adenine dinucleotide (NAD: NADH) ratio. To sum up, AMPK influences the aging process through a variety of pathways [51].

A large number of researches have demonstrated that AMPK plays an anti-aging role through histone methyltransferase. AMPK phosphorylates the histone methyltransferase EZH2 at T311 [52]. Moreover, it has been reported that AMPK activator metformin leads to increased trimethylation of H3K79 and regulates mitochondrial biogenesis and senescence through H3K79 methylation [53]. AMPK-mediated phosphorylation resulted in the activation of histone acetyltransferase 1(HAT1) [54]. H3K4me3 antagonizes the HIR/Asf1/Rtt106 repressor complex to promote histone gene expression and extend chronological life span. Our GO functional analysis suggesting that metformin affects the activity of histone methyltransferase (H3K9 specific). A new research indicated that changes in AMPK phosphorylation following changes in histone 3 (H3K9) acetylation and methylation status [55]. Therefore, based on our research, metformin may affect the DNA methylation status histone 3 (H3K9) acetylation thus inhibiting AMPK and performing its anti-aging effect.

4.4 Functional roles of genes associated with the DMPs

Then, we analyzed the main known functional roles of genes associated with the DMPs. The identified CpG sites respond to 7 genes. The seven genes were EPM2AIP1, MUC17, FNDC1, HLA-DOB, TRPC4, ESRRB, ADCY2. These genes have previously been reported to be associated with aging. For example, the

absence of EPM2AIP1 in mice increases liver fat, causes hepatic insulin resistance, and protects against age-related obesity [26]. It has been reported that endothelial TRPC4 mRNA levels were apparently decreased in aging rats [27]. FNDC1 is associated with human skeletal muscle aging [28]. However, the relationship between these genes and aging remain unreported to date, such as cg23858082 (MUC17), cg04348707 (HLA-DOB), cg07730622 (ESRRB), cg03739343 (ADCY2). Although the functional impact of differential methylation in the specific genes associated with metformin medication remains to be established, identification of several unreported functions of several genes make sense biologically.

Limitations

Although we demonstrated the metformin slow down the epigenetic age of the DM patients and metformin is a potentially promising anti-aging drug, there still some limitations of our study. The sample size is relatively small and limited to a single center. There still some lifestyle factors that can influence DNA methylation, such as any potential difference between diet and physical activity levels among the groups. Although all of the participants reported that they were on a normal diabetic diet, we know the Chinese diet is complex and these factors cannot be compared in most cases. Because We try to avoid the effects of diet on DNA methylation levels to some extent.

Conclusions

Our result demonstrated the metformin slow down the epigenetic age of the DM patients thus performing its anti-aging effect.

List Of Abbreviations

AMPK	Adenosine activated protein kinase
DM	Diabetes Mellitus
DMPs	differential methylation positions
mTOR	mammalian target of rapamycin
BMI	body mass index
CpGs	cytosine–guanine dinucleotides
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Board of the Chinese PLA General Hospital. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Written informed consent for publication was obtained from each author and each patient.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Hongbin Liu contributed to substantial contributions to the conception or design of the work. Man Li contributed to data collection, data interpretation, and critical review of the manuscript drafting the manuscript. Lei Duan, Yulun Cai, Benchuan Hao, Jianqiao Chen contributed to data collection. All authors read and approved the final manuscript.

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Figures

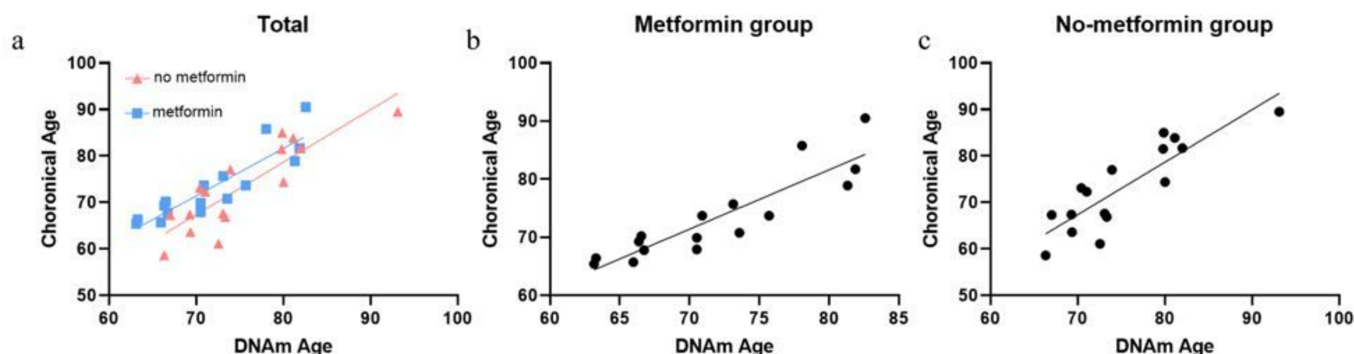

Figure 1

Correlation between epigenetic age and chronological age

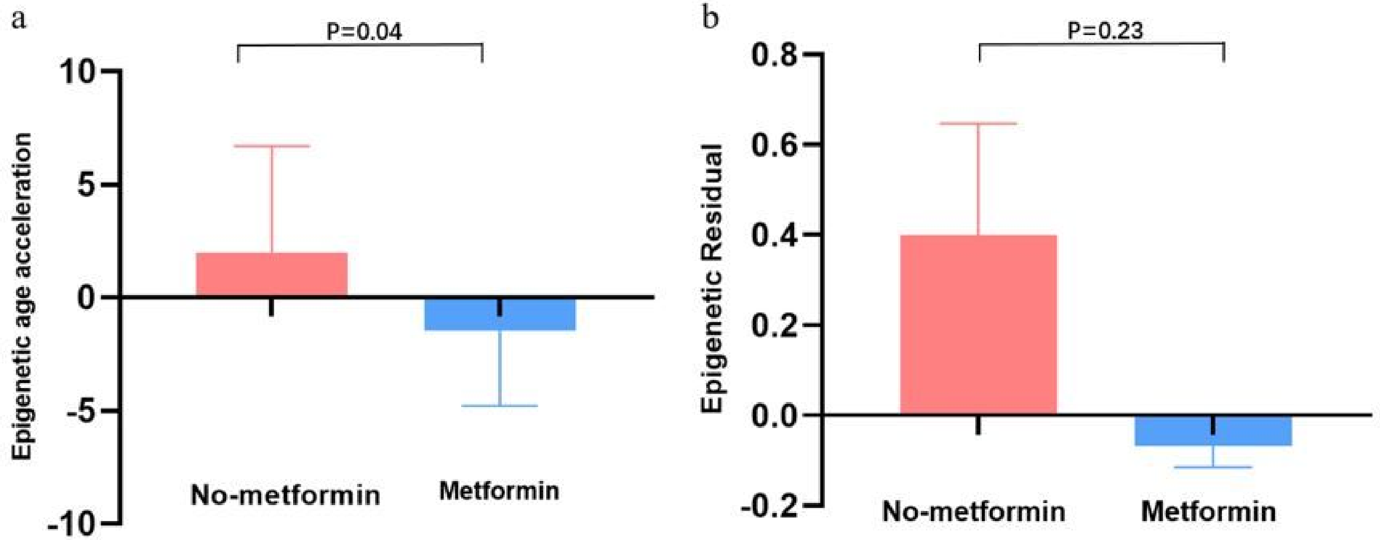

Figure 2

Acceleration Diff, and Acceleration Residual value of the two groups

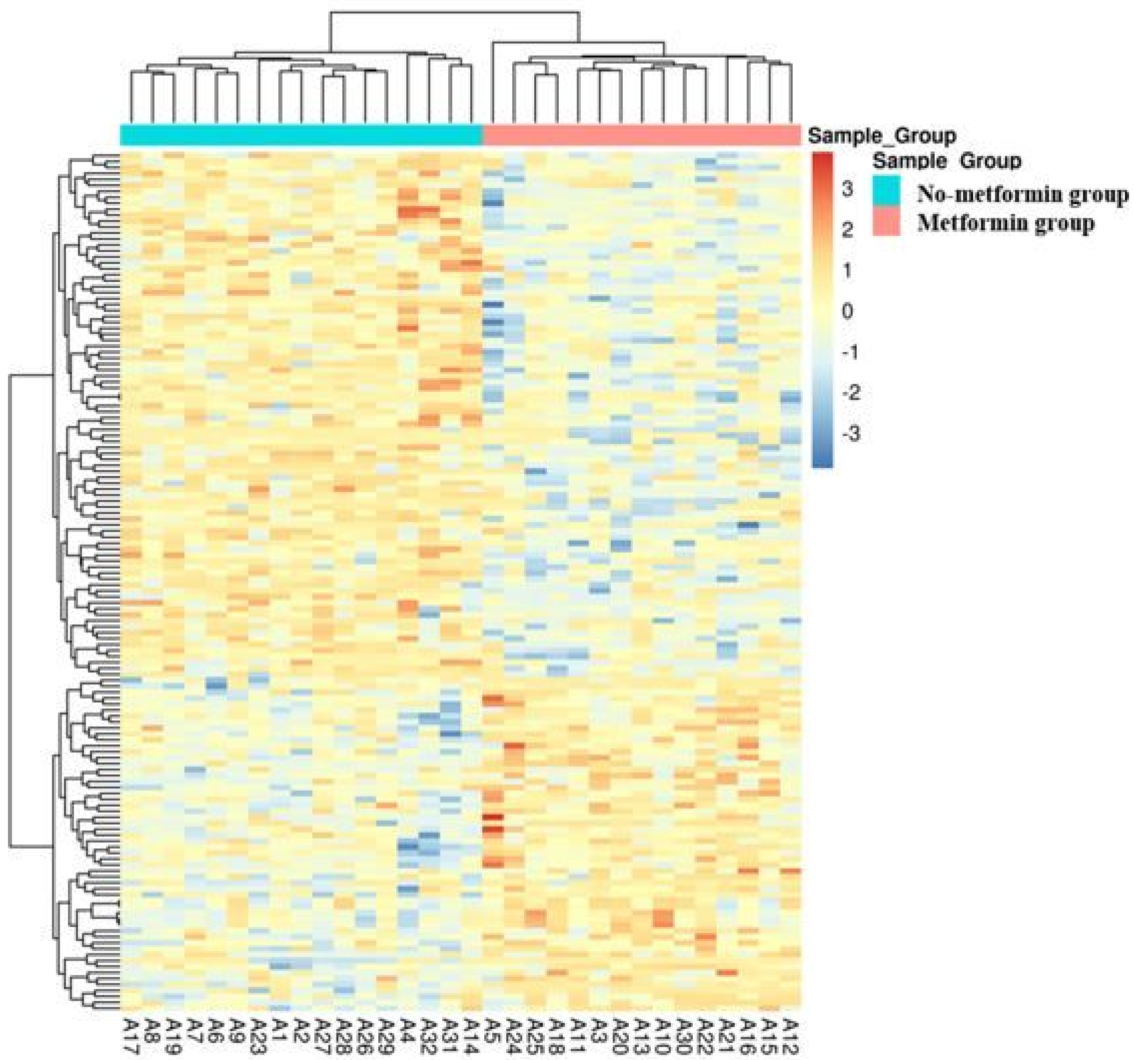

Figure 3

Heat map of the two groups

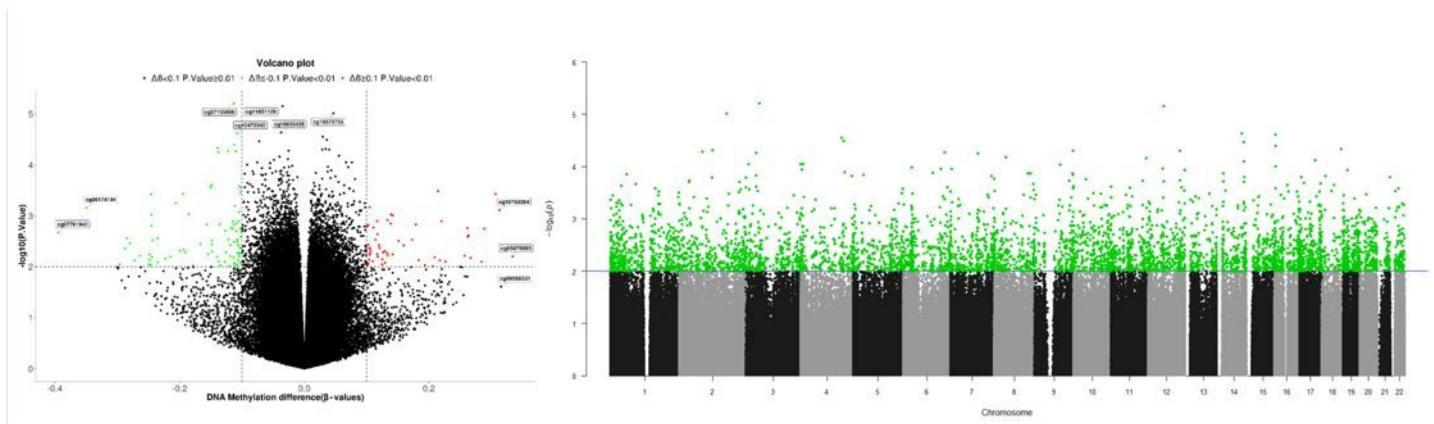

Figure 4

Volcano spot shows the significantly differentially methylated between the metformin group and no-metformin group and their distribution in chromosome

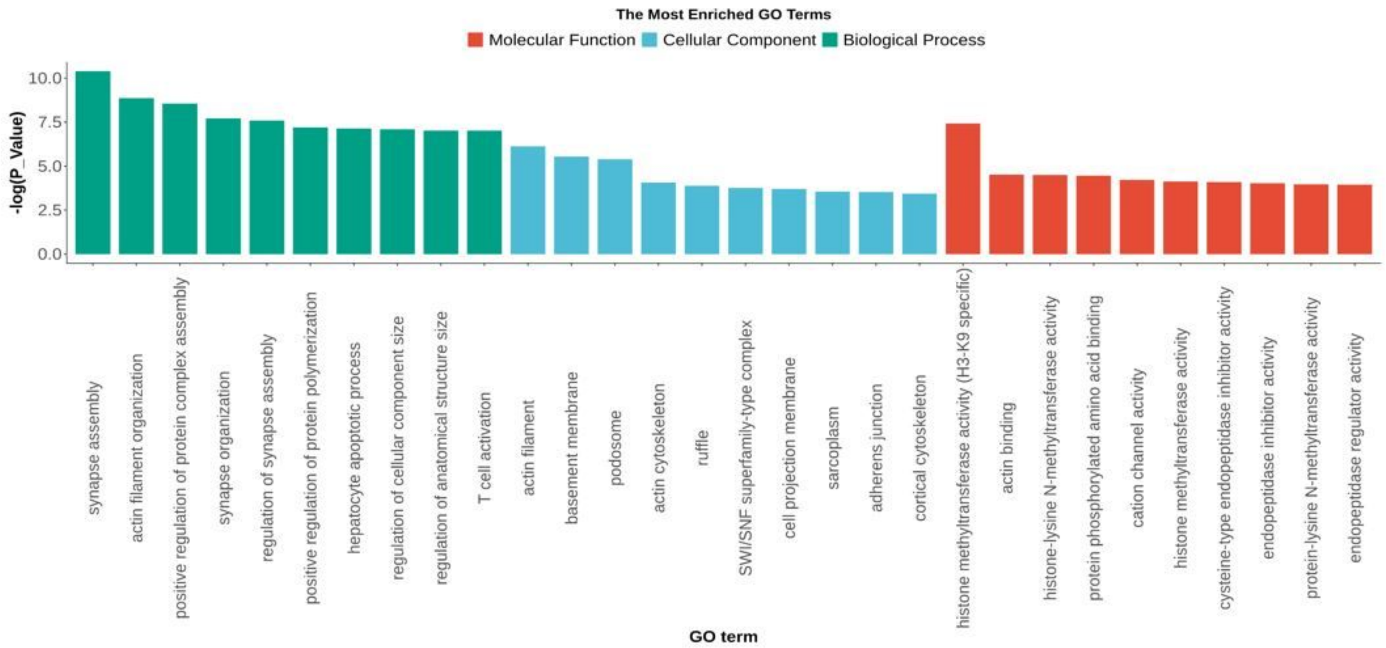

Figure 5

GO enrichment analysis