Department of Clinical Pharmacology[1], Xiangya Hospital, Institute of Clinical Pharmacology[2], Central South University, Hunan Key Laboratory of Pharmacogenetics; Changsha; Department of Cardio-Thoracic Surgery[3], the Second Xiangya Hospital of Central South University, Changsha; Key Laboratory of Bio-resources and Eco-environment[4] (Ministry of Education), College of Life Science, Sichuan University, Chengdu, China

Identification of gene modules associated with warfarin dosage by a genome-wide DNA methylation study

Zhiying Luo[1,2], Rong Liu[1,2], Bao Sun[1,2], Xinming Zhou[3], Zhaoqian Liu[1,2], Honghao Zhou[1,2], Heng Xu[4], Xi Li[1,2,*,#], Wei Zhang[1,2,*,#]

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**Corresponding authors: Prof. Wei Zhang, Xi Li, Department of Clinical Pharmacology, Xiangya Hospital, Central South University, 110 Xiangya Rode, Kaifu district, Changsha, Hunan 410008, China*
yjsd2003@163.com; lixi6931430@126.com
#Wei Zhang and Xi Li contributed equally to this study.

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Objective: To identify warfarin dose-associated DNA methylation changes, we conducted the first genomewide DNA methylation association study. *Method:* A total of 22 patients who required an extreme warfarin dosage from *VKORC1* -1639AA & *CYP2C9**1*1 genotype group were enrolled in this study. The Illumina Infinium HumanMethylation450 platform was used to perform genome-scale DNA methylation profiling, identifying differentially methylated CpG sites by a nonparametric test. WGCNA was used to analyze the association between gene modules and extreme warfarin dosage. *Results:* For a total of 378,313 CpG sites that passed the quality control processes, we identified eight differentially methylated CpG probes (p<0.05) showing altered DNA methylation level (>20%) between two extreme dose groups. Though the WGCNA method we identified two gene modules, Turquoise and Light-cyan, with high methylation level were significantly correlated with high warfarin doses (P-values were 0.036 and 0.022 respectively). Both gene modules exhibited good warfarin dosage prediction performance (77% for the Turquoise module and 79% for the Light-cyan module). *Conclusion:* This study showed for the first time that DNA methylation level changes are significantly associated with warfarin dosage, providing a novel idea for understanding warfarin dose various and laying the groundwork for further related studies.

1. Introduction

Warfarin is typically the first choice of the oral anticoagulants available to reduce the risk of thromboembolic disorders, but it is also the drug with the highest hospital readmission rate (Lee and Klein 2013). The required warfarin dose depends on both clinical and genetic factors. Polymorphisms of the warfarin's target gene (*VKORC1*) and the gene encoding the main enzyme that metabolizes warfarin (*CYP2C9*) have been be identified as the primary reason for variability in the required dose (Klein et al. 2009). Several retrospective studies have focused on the development of genetically based warfarin dose prediction algorithms to facilitate individualized administration. However, the clinical applicability of these algorithms is still under evaluation, because of different conclusions drawn in several prospective trials with large sample sizes (Kimmel et al. 2013; Verhoef et al. 2013; Tan et al. 2012). Based on these studies, we recently conducted a two-stage extreme phenotype design to explore novel mutation associated with differences in the required warfarin dose (Luo et al. 2017). However, the newly identified mutations play a limited role in explaining warfarin dose variations. Therefore, we further hypothesized that epigenetic factors might also contribute to the differences in warfarin therapeutic dose.

'Epigenetic' refers to post-translational modifications of histone proteins, DNA methylation, chromatin remodeling and noncoding RNAs (Berger et al. 2009). Recent studies have shown that epigenetic processes not only regulate the expression of genes that are responsible for the development of diseases but also affect drug treatment by modulating the expression of key genes involved in the absorption, distribution, metabolism and excretion (ADME) of drugs, thereby contributing to inter-individual variations in drug response and adverse drug reactions (Gomez and Ingelman-Sundberg 2009; Liu et al. 2014; Fidel et al. 2016). DNA methylation is one of the most common epigenetic modifications that influence gene expression. We therefore proposed that alternations in the DNA methylation of genes that participate in the disposition of warfarin might explain warfarin dose differences.

As the *VKORC1 -1639*AA and *CYP2C9* *1*1 genotype had the highest frequency in Chinese population Zeng et al. 2012), and warfarin dose differences of patients with this genotype can rarely be explained by the polymorphisms *VKORC1* and *CYP2C9* gene. In this study, we used an extreme phenotype strategy to analyze the genome-wide DNA methylation difference in 22 patients required extreme warfarin doses, and with the *VKORC1* -1639AA & *CYP2C9**1*1 genotype. We sought to find altered methylation sites associated with extreme differences in warfarin dose requirements.

2. Investigations and results

2.1. Epigenome genome-wide analysis

The clinical characteristics of the 22 patients in the wild-type group who required an extreme warfarin dose are described in Table 1. The warfarin dose of the high dose group was much higher

Table 1: Baseline characterise between patients

Variables	High dose patients	Low dose patients	P-value
WSD, mg/day	4.34±0.44	1.35±0.15	0.000
Sexual, M/F	1/10	2/9	0.56
Age, year	45.63±8.5	53.36±8.94	0.051
High. cm	156.81±5.76	157.18±7.45	0.899
Weight, kg	58.59±7.43	53.18±5.72	0.07

Fig. 1: Epigenetic-wide association between warfarin high dose patients and warfarin low dose patients of 378313 CpGs. X-axis indicates chromosome position and y-axis indicates $-\log_{10}$ of P-value for each CpG site.

than that of the low dose group (4.34±0.44 mg/day *vs.* 1.35±0.15 mg/day, P-value <0.001), and the clinical indexes were matched between the two patients groups (P-value > 0.05). After preprocessing and QC analyses, 378,313 CpG sites were retained for further analysis. An epigenome-wide analysis of these CpG sites was conducted to identify warfarin dose associated DNA methylation sites. The $-\log_{10}$ values (p-value) of each site were plotted across the genome (Fig. 1).

Hierarchical clustering of the top 50 statistical difference probes that varied the high dose group patients and the low dose group patients was performed. On the whole, the DNA methylation profiles of the high dose group and the low dose group resulted in separate clusters, indicating a substantial difference in DNA methylation profiles between high and low warfarin dose patients (Fig. 2). We then performed a locus-by-locus differential DNA methylation analysis using a cut-off of $p < 0.05$ and a minimum median β-value difference of 20%, identifying 3 probes with significant hypomethylation and 5 probes with significant hypermethylation in extreme low dose groups (Fig. 3, Table 2).

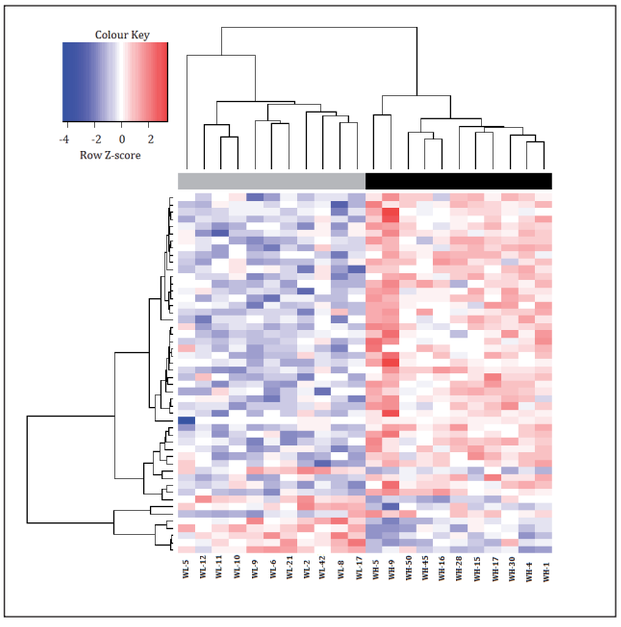

Fig. 2: Hierarchical cluster analysis of top 50 most significant DNA methylation sites associated with extreme warfarin dose. Notice the perfect segregation between the extreme high and low warfarin dose patients.

2.2. *Identification of gene models associated with warfarin dose*

The WGCNA method was used to evaluate the relationship between gene-network methylation and required warfarin dose difference. A total of 17 gene sets were first identified in these 11 pairs of patients receiving extreme warfarin dose, as shown in Fig. 4. The methylation level of each gene was calculated by the methylation level of the CpG sites in the gene, and the association between these gene modules and warfarin dose was shown in Fig. 5A. In addition, the turquoise and light-cyan modules had higher mean significance than the other modules. Univariate associations between the gene modules and warfarin dose were analyzed (Table 3), high model scores of turquoise and light-cyan modules were significantly associated with warfarin dose (P value < 0.05). Both the turquoise and light-cyan modules had high methylation level in high dose group, as shown in Figs. 5B and 5C.

2.3. *Comparing the prediction accuracy by ROC analysis*

The ability of the turquoise and light-cyan modules to predict warfarin extreme is shown in Fig. 6. Both the Turquoise and Light-

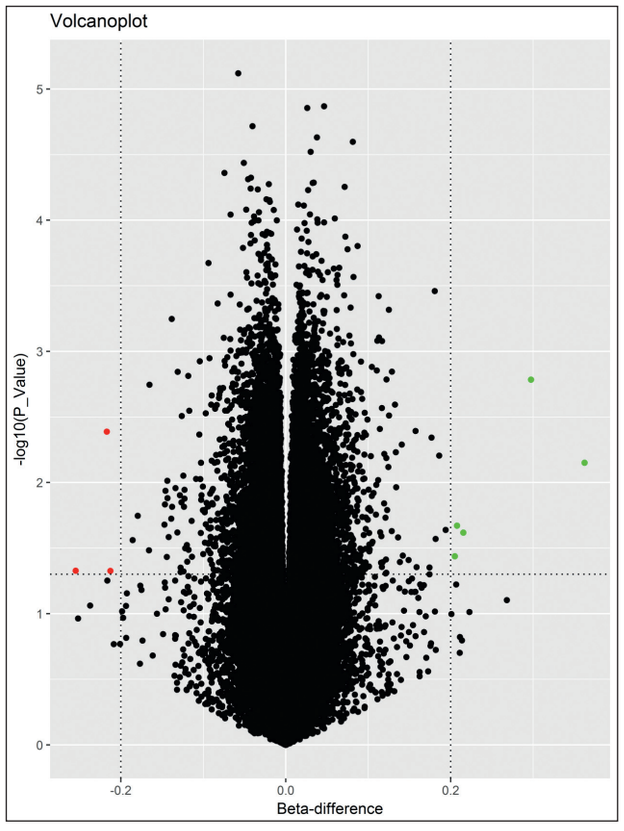

Fig. 3: Differentially methylalted CpG probes associated with extreme warfarin dose.
Note. Beta-difference represents the difference in mean methylation between high and low dose patients. The y-axis (-log10 (P-value)) represents the negative log of the association p-value.

cyan modules had high prediction accuracies, with AUCs of 77% and 79% respectively. Both modules successfully separated the high-dose patients from low-dose patients.

Table 3: Univariate associations between gene modules and warfarin dose were analyzed

Module	Wilcoxon rank sum test p value
Blue	0.65
Red	0.47
Grey60	0.43
Salmon	0.84
Black	0.79
Greenyellow	0.10
Brown	0.65
Purple	0.36
Magenta	0.43
Midnightblue	0.56
Yellow	0.26
Pink	0.95
Green	0.17
Cyan	0.90
Lightcyan	0.022
tan	0.15
Turquoise	0.036

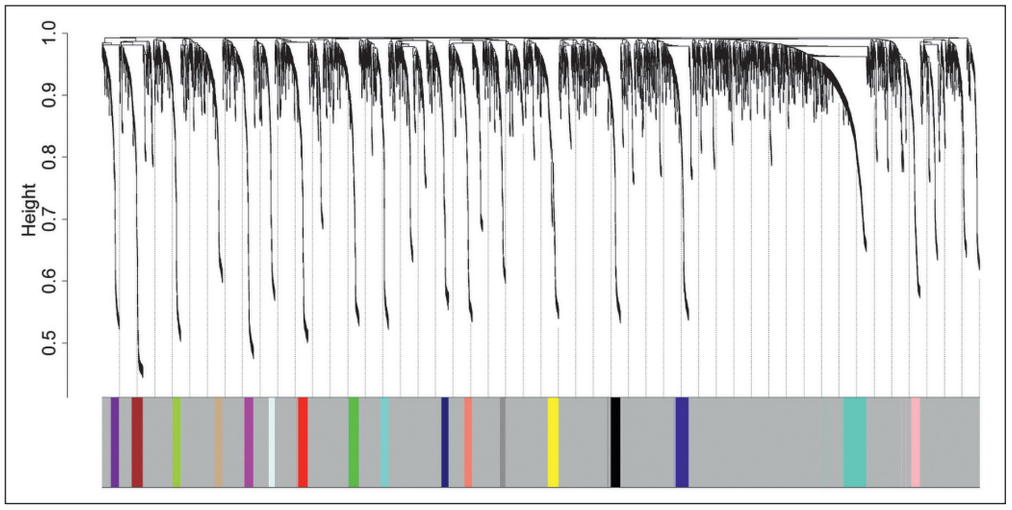

Fig. 4: Gene sets identified by the WGCNA method.
Note. This dendrogram was building based on data of 22 warfarin extreme dose patients. Each line on the figure represent one gene, and the branch mean high co-expression gene set. Each colour of the bottom represent a gene set, but the Grey represents this gene was not assigned to any gene set.

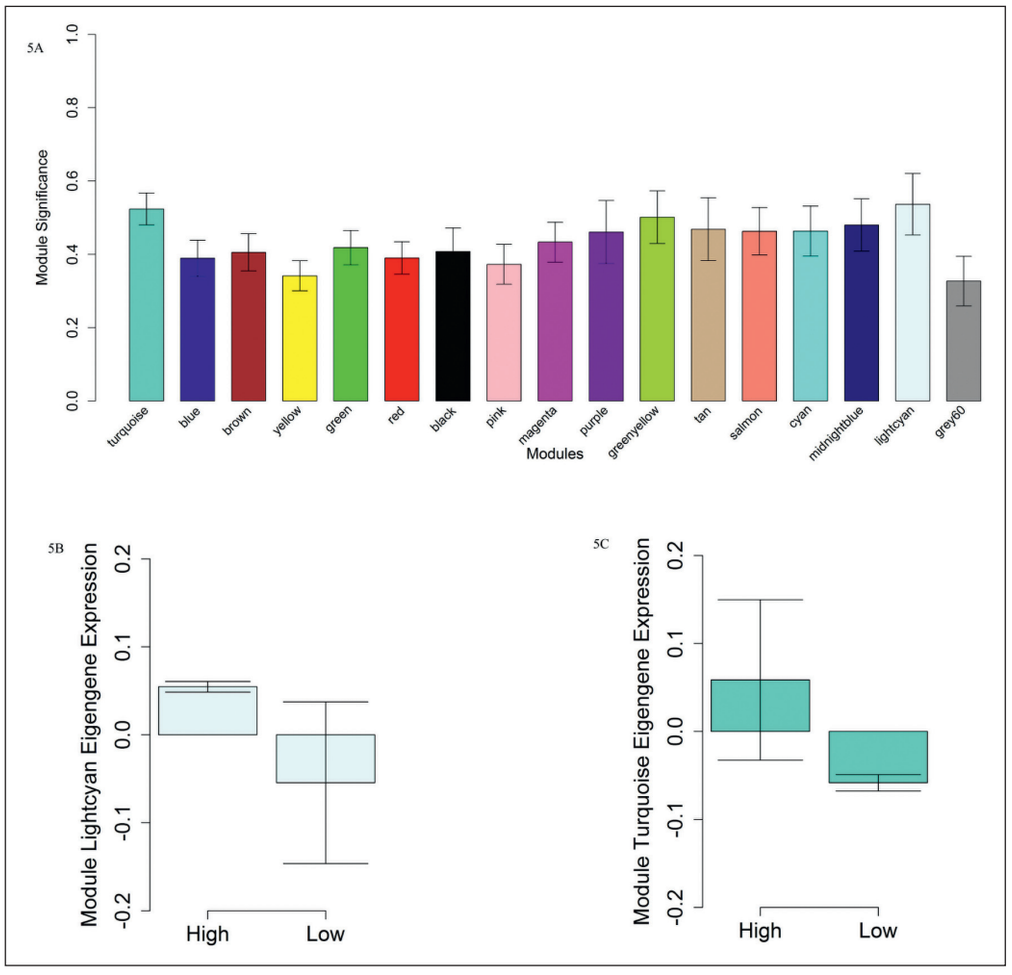

Fig. 5: Association between warfarin dosage and gene modules.
Note: 3A: X-axis: each gene module was represented as a stained-pillar; Y-axis: the module significance (MS). MS was calculated based on two step: 1) calculated the p-value of each CpG site by wilcoxon rank sum test, and the P-value of each site was further translated to gene significance (GS) (-log(p value)); 2) the mean of the GS values of genes in each gene module means MS. 3B and 3C were the relationship between the Turquoise and Light-cyan modules and extreme warfarin dose respectively.

Table 4: List of the GO term in the significant DAVID functional cluster for the Turquoise gene module

Term	Count	%	PValue	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
GO:0007156~homophilic cell adhesion	12	2.03	8.66E-12	PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHA2, PCDHA3, PCDHA4, PCDHB4, PCDHA5, PCDHA1, CDH13, PCDHA10, PCDHA11, PCDHA12, PCDHA13	20.65	5.43E-09	5.43E-09	1.29E-08
GO:0016337~cell-cell adhesion	14	2.36	1.40E-10	PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHA2, PCDHA3, PCDHA4, PCDHB4, PCDHA5, PCDHA1, SOX9, CDH13, COL14A1, PCDHA10, PCDHA11, PCDHA12, PCDHA13	11.44	8.79E-08	4.40E-08	2.08E-07
GO:0007155~cell adhesion	16	2.70	2.09E-07	PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHA2, PCDHA3, PCDHA4, PCDHB4, PCDHA5, CPXM2, MSLNL, PCDHA1, SOX9, CDH13, COL14A1, PCDHA10, PCDHA11, PCDHA12, PCDHA13	5.15	1.31E-04	4.36E-05	3.10E-04
GO:0022610~biological adhesion	16	2.70	2.12E-07	PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHA2, PCDHA3, PCDHA4, PCDHB4, PCDHA5, CPXM2, MSLNL, PCDHA1, SOX9, CDH13, COL14A1, PCDHA10, PCDHA11, PCDHA12, PCDHA13	5.15	1.33E-04	3.33E-05	3.16E-04

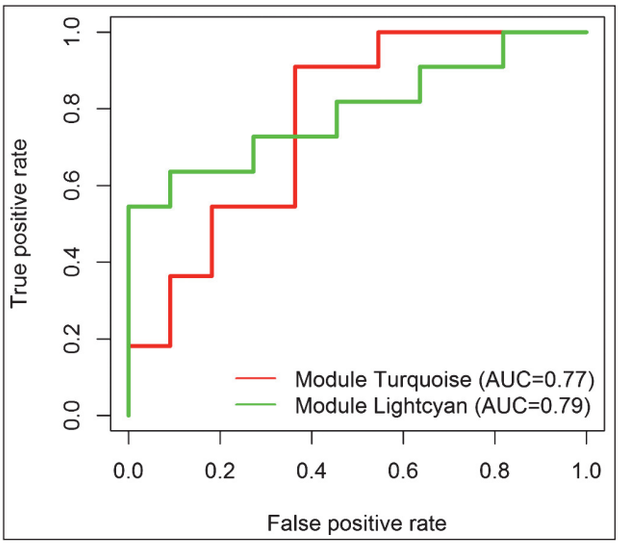

Fig. 6: Comparison of the prediction ability of two models.

2.4. Gene ontology and pathway analyses

To investigate the function of these two modules, a DAVID gene functional annotation analysis was performed. GO biological processes showed that the Turquoise module contained genes enriched in cell adhesion (Table 4). Genes in the lightcyan module were not significantly associated with any specific processes.

3. Discussion

Using a genome-level interrogation of DNA methylation, we performed the first reported genome-wide analysis of DNA methylation profiles to explore the role of DNA methylation in warfarin dose differences. In this study, we identified eight differential methylations sites and two gene modules associated with warfarin dosage, which may be helpful in developing new biomarkers for warfarin dose prediction.

An extreme phenotype strategy was used to find warfarin dose-associated methylation differences in a small sample size, based on the assumption that gene functional changes that usually occur at a very low frequency would be present more frequently in patients with extreme phenotypes Ramsey et al. (2012). Previous studies have focused on finding novel warfarin dose-associated mutations and establishing pharmacogenetic warfarin dose prediction models (Perera et al. 2013; Parra et al. 2015). However, the effect of genome-wide DNA methylation on variability in required warfarin doses has not been examined. A previous study showed that DNA methylation at the CpG islands of the VKORC1 promoter did not correlate with VKORC1 mRNA expression or warfarin dose variation (Wang et al. 2008). However, there is also evidence that DNA methylation of CYPs (CYP1A, CYP1A2, CYP2C9, CYP3A4), which participate in the warfarin metabolism process, is associated with the mRNA expression of these genes (Ingelman-Sundberg et al. 2007). In addition, we previously demonstrated that polymorphisms of the DNA methyltransferase DNMT3A, which catalyzes the predominant epigenetic modifications in human, were significantly associated with warfarin dosage (Luo et al. 2017).

In this study, we identified eight probes with significant methylation change associated with extreme warfarin dosage using a cut-off of $p < 0.05$ and a minimum median β-value difference of 20%. Four of these eight probes located in B3GNT3, ADARB2, FLG2 and KCTD5 genes, respectively, but no definite proof can link the relationship between these genes and warfarin dose requirement. In this study, we did not identify a CpG site that was significantly associated with warfarin dosage after correction or using of p-value threshold (p-value<10-7).

WGCNA incorporates information from all probes in a module for evaluating the modules' relationship with a phenotype. Our final results showed that two gene modules related to cell adhesion were significantly associated with warfarin extreme dose. There is evidence that warfarin could reduce the action of an extrahepatic Gla-protein (Gas6), which has a broad range of regulatory functions associated with cell growth, migration and proliferation, cell survival, apoptosis, phagocytosis and cell adhesion (Park et al. 2009). The anti-adhesion effects of warfarin and heparin have also been described as prolonging the circulation period of cancer cells in the blood stream by disrupting cancer cell adhesion to the endothelium and platelets and making the tumors more vulnerable to the cytotoxic action of NK cells (Bobek and Kovarik 2004; Bobek et al. 2003). An in vivo research has shown that a high warfarin dose results in increased responsiveness of granulocytes to PMA stimulation of adhesion in rats (Belij et al. 2012). A recent study showed that warfarin inhibits cell adhesion through inhibiting epithelial cell adhesion molecule (EpCAM)-mediated cell-cell adhesion by reducing the expression of EpCAM protein (Shao et al. 2016).

The effective anti-adhesion role of warfarin has been demonstrated in various cell modes, and these results have broadened the clinical use of warfarin. However, the influence of cell adhesion on

warfarin dosage was firstly studied by us. As a high methylation level is usually associated with low gene expression, we can hypothesize that a low cell adhesion level is associated with the requirement of high warfarin doses.

There are some limitations in this study. First, the drug targeting and metabolism processes of warfarin occurs in the liver, but we analyzed the methylation level of DNA from peripheral blood. only. Second, these identified warfarin dose associated gene modules were not further verified using genome-wide DNA methylation chips. Third, though we using the extreme phenotype strategy to select representative samples, the sample size in our study was small and the results need further validation.

4. Experimental

4.1. Samples and study design

We previously enrolled 1617 patients on a stable warfarin dosage regimen, of whom 943 exhibited VKORC1 -1639AA and CYP2C9*1*1 genotype (designated as the wild-type group). In this study, we specifically enrolled 11 paired patients from the wild-type group receiving extreme high or low therapeutic warfarin doses. The clinical characteristics of these 22 patients were matched. All of these patients collected blood samples before warfarin treatment. DNA methylation was assessed across the whole genome in each of the 22 individual samples using the Illumina 450K BeadChip.

4.2. DNA extraction and bisulfite conversions

Total genomic DNA was extracted from whole blood samples (2 ml) from each participant using a Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA, Catalog: A1125) and converted using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions.

4.3. DNA methylation data production and normalization

The converted DNA was assessed using the Illumina Infinium HumanMethylation450 BeadChip arrays (Illumina, San Diego, CA). All samples were processed in a single working batch and hybridized on BeadChips. Each chip was scanned in a HiScan 2,000 (Illumina). This array generates DNA methylation data for >480,000 CpG sites in 99% of RefSeq genes. The methylation levels were computed as β values, calculated from the mean methylated (M) and unmethylated (U) signal intensities for each probe for each sample using the formula (β=M/M+U+100).

The normalization steps were performed using the Bioconductor package. Stringent filtering of the Illumina HM450K data was conducted: CpG sites containing missing values (n=4,926), cross-reactive probes and polymorphic CpGs (n=91,730), and CpG sites located in the X or Y chromosome (n=10,549) were removed. Probes with a p-value <0.05 were considered to be statisticatly different between the tested groups, 357 sites had at least 75% of samples with a p-value great than 0.05 and were removed. In total, leaving 378,313 sites were retained from the original 485,577 sites for further analysis.

4.4. Weighted gene co-methylation network analysis

Weighted gene co-methylation network analysis (WGCNA) is used for the hierarchical clustering of correlated methylation sites to construct weighted co-methylation modules, and the eigengene of each module mathematically summarizes the co-methylation information of all probes within each module for modeling purpose. The R 'WGCNA' package was used to analyze network modules. In this study, scale-free properties were achieved with a threshold of 6, resulting in 17 modules. The co-methylation dissimilarity for each gene pair from the adjacency matrix was determined via a network distance measure known as the topological overlap measure. Extreme warfarin dose associated gene modules were limited to genes with a stringent module membership cutoff of > 0.9 by the hybrid dynamic tree cutting method. The association between each gene module and an extreme warfarin dose was analyzed by a Wilcoxon rank sum test.

4.5. Gene ontology analyses

Differentially methylated gene modules underwent Gene Ontology (GO) terms and KEGG pathway analysis using the DAVID Functional Annotation Tool. All genes measured by the HM450 were used as the reference set in this analysis.

4.6. Statistical analysis

Continuous data (presented as the mean and standard deviations) were compared using Student's t test or non-parametric tests, with Fisher's exact tests and binary logistic regression applied in the comparison of categorical data between groups. The R version 2.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 20.0 software (IBM, SPSS, Chicago, IL, USA) were used for the statistical analyses, with differences considered to be statistically significance at p<0.05.

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Contribution: Wei Zhang, Xi Li and Zhiying Luo designed the experiments. The data collection, DNA extraction and genotyping works were accomplished by Zhiying Luo and Bao Sun. Zhiying Luo and Xi Li drafted the manuscript. All of the patients were enrolled under the help of Xinming Zhou, Guobao Song and Xiaobin Li. The statistic works were conducted under the guidance of Xi Li and Rong Liu.

Conflict of interest: The authors have no conflicts of interest to declare.

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