RESEARCH ARTICLE

WILEY

DNA methylation profile in the whole blood of acute coronary syndrome patients with aspirin resistance

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Abstract

Background: Aspirin resistance (AR) results in major adverse cardiovascular events, and DNA methylation might participate in the regulation of this pathological process.

Methods: In present study, a sum of 35 patients with AR and 35 non-AR (NAR) controls were enrolled. Samples from 5 AR and 5 NAR were evaluated in an 850 BeadChip DNA methylation assay, and another 30 AR versus 30 NAR were evaluated to validate the differentially methylated CpG loci (DML). Then, qRT-PCR was used to investigate the target mRNA expression of genes at CpG loci. Finally, Gene Ontology (GO) as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to reveal the enriched pathways.

Results: The AR and NAR groups displayed significant differences in DNA methylation at 7707 positions, with 270 hypermethylated sites (e.g., cg09555818 located in APOC2) and 7437 sites hypomethylated sites (e.g., cg26828689 located in SLC12A5). Six DML were validated by pyrosequencing, and it was confirmed that DNA methylation (cg16391727, cg21008208, cg21293749, and cg13945576) was related to the increasing risk of AR. The relative mRNA expression of the *ROR1* gene was also associated with AR ($p = 0.007$), suggesting that the change of cg21293749 in DNA methylation might lead to differential *ROR1 mRNA* expression, ultimately resulting in AR. Furthermore, the identified differentially methylated sites were associated with the molecular pathways such as circadian rhythms and insulin secretion.

Qinglin Yu, Jin Yang, Jiang Wang are the equal contributors to this article and they are first co-authors.

Conclusion: Hence, the distinct DNA methylation might play a vital role in the biological regulation of AR through the pathways such as circadian rhythms.

KEYWORDS
acute coronary syndrome, aspirin resistance, circadian rhythms, DNA methylation, ROR1

1 | INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, with an incidence of 10% in people over 60 years old, and it is gradually increasing in young and middle-aged people.[1] Antiplatelet therapy is recognized as a fundamental preventive measure to reduce adverse cardiovascular events, especially in acute coronary syndrome (ACS) patients after percutaneous coronary intervention (PCI). However, approximately 4% of cardiovascular adverse events still reoccur every year, despite rational standard antiplatelet strategies.[2] Due to the weak responses or nonresponsiveness of some individuals to aspirin, the pathological process of platelet aggregation cannot be successfully prevented, which is defined as aspirin resistance (AR).[3,4] A systematic meta-analysis involving more than 10 thousand patients showed that the AR prevalence in CVD patients was 24%.[5] Another study revealed that AR was significantly associated with major adverse cardiovascular events (relative risk of 2.09).[6] Because of this phenomenon, the safety and efficacy of platelet inhibition have gained the utmost importance in recent decades.

Previous researches have suggested the AR's mechanisms includes clinical factors, comorbidities, and genetic factors. Clinical factors, including PCI history,[7] platelet counts,[8] male sex, smoking and obesity,[9] are independent risk factors for AR. With regard to comorbidities, primary underlying diseases such as hypertension,[10] diabetes,[11] cancer, severe coronary atherosclerosis, chronic kidney disease, and hyperlipidemia[12] lead to a low aspirin responses in individuals. The increase in oxidative stress in these diseases may promote platelet activation and reduce the efficacy of aspirin. In regard of genetics, study on AR is mainly concentrated on single nucleotide polymorphisms (SNPs). SNPs in platelet receptor genes (GP IIIa,[13] GP IV,[14] GP Ibα,[15] PEAR1,[16] P2Y12, and P2Y1[17]), platelet activation-related genes (HO-1,[18] TXB2,[19] COX-1 and COX-2[20]), drug absorption and metabolism genes (CYP2C19,[21,22] ABCB1,[23] UGT1A6[24]), and lipid metabolism genes (ApoE[25]) have been proven to be associated with AR. Accordingly, the interactions between gene SNPs is also a factor that cannot be ignored.

The genetic study of pathogenic mechanisms is a hot spot of current research, ranging from mechanisms involving the above SNPs to epigenetics. Epigenetics is closely related to the occurrence and development of pathological state such as congenital heart disease.[26] Noncoding RNAs (including miRNAs lncRNAs and circRNAs), histone modifications, and methylation of DNA and RNA are the major components of epigenetic patterns. Previous research by our team identified an effect of the ABCB1,[27] P2Y12,[28] and PON1 genes[29] on clopidogrel resistance (CR) and revealed DNA methylation profile in the whole blood of CR individuals[30] through microarray analysis. However, DNA methylation changes in AR patients are rarely reported. Alterations in DNA methylation modifications would influence the target genes' expression, resulting in the diverse antiplatelet effects to aspirin.

With the wide clinical use of aspirin, it is essential to explore the molecular mechanisms and genetic methylation risk factors for AR. The signaling pathways involved in the regulation of weak aspirin reactions are fully explored in the present study through the identification of various methylated CpG loci and pathway enrichment analysis, clarifying the causes of low drug reactivity in some patients.

2 | MATERIALS AND METHODS

2.1 | Study population

A sum of 35 aspirin resistance (AR) patients and 35 non-aspirin resistance (NAR) controls were enrolled in present research from October 2020 to December 2021; all subjects were recruited from the Ningbo First Hospital and belonged to the Han Chinese population of Ningbo. All patients and controls were older than 18 years and were ACS patients who had undergone PCI (treated with a drug-eluting stent). The diagnostic criteria for AR should met followings: including clinical confirmation of aspirin antiplatelet therapy failure and an aspirin reaction unit (ARU) >550,[31,32] measured by the VerifyNow P2Y12 assay (Accumetrics, Inc.). Neither the patients nor the controls had severe infections, hepatic or kidney function disorders, a history of active bleeding, or rheumatoid-related diseases. After informed consent was provided by the patients or their guardians, the blood samples and clinical data were obtained. Complied with the Declaration of Helsinki, the study protocol was carried out based on appropriate ethical procedures supported by the Ethics Committee of Ningbo First Hospital.

2.2 | DNA extraction

Blood samples from 35 AR patients and 35 NAR subjects were collected, 10 of which were used for 850 BeadChip DNA methylation assay, and the remaining samples (30 AR and 30 NAR) were prepared

for validating the DNA microarray results. Blood samples were stored frozen in a −80°C freezer.

The DNA purified from peripheral blood was extracted with a QIAamp DNA Blood Mini Kit (Qiagen). The DNA concentration and purity were quantified in a NanoDrop 2000 system (NanoDrop) by measuring the optical density ratio of the maximal absorbent wavelengths from 260 to 280nm (OD260/280). The DNA concentration was required to be higher than 50ng/μl, and the OD260/280 ratio had to fall between 1.60 and 2.10.

And then each qualified sample was transferred to a centrifuge tube according to the kit instructions and placed in a −20°C freezer. Agarose gel electrophoresis with ethidium bromide staining was used to determine the extracted DNA integrity.

2.3 | 850 BeadChip DNA methylation assay

Whole-DNA methylation was assessed with the Infinium Human Methylation 850 BeadChip Kit (Illumina, Inc.), which covered 853,307 cytosine positions in the human genome. A whole train of steps were operated for the processing of DNA samples according to the provided instructions. The DNA samples first underwent DNA denaturation, followed by whole-genome amplification, fragmentation, precipitation and resuspension, and hybridization to arrays.

2.4 | Pyrosequencing and validation of differentially methylated CpG loci (DML)

For confirming the DNA microarray results, six CpG loci with different degrees of DNA methylation (cg16391727, cg21463518, cg21008208, cg21293749, cg13945576, and cg13703859) were selected for cross-validation in 30 AR patients and 30 controls. Thus, each blood DNA sample from both groups was double-vulcanized in line with the manufacturer's instructions. We used the PyroMark PCR kit (Qiagen) to perform PCR amplification of the region of interest. The applied nucleotide probes included a biotinylated version allowing detection with streptavidin Sepharose (Table S1). We purified and further performed the single-stranded DNA that had been biotinylated.

The above six differentially methylated loci were located in the genes of *PRKAG2*, *ROR1*, and *ROR2*. We validated the relative mRNA expression of these three genes through qRT-PCR. The RNA was extracted via RNeasy Plus Universal Kit (Qiagen). The PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa Bio) was applied to synthesize cDNA from 1 μg of RNA. Template cDNAs were diluted 1:4, and an ABI 7500 Quantitative Real-time PCR (qRT-PCR) System (Applied Biosystems) was used to quantify the gene expression. The housekeeping gene GAPDH was utilized as the internal control. The primers were designed by Primer Premier 5 and the sequences of the PCR primers were listed in Table S2. After each sample was measured in triplicate, the average value was taken. The mRNA expression of these three genes were assessed with the relative quantitative method.

2.5 | Statistical analysis

The statistical analysis was performed by SPSS 20.0. The distribution of variables was tested with the Kolmogorov–Smirnov test. Means of designated comparison groups were compared with *t*-test or a nonparametric test. A two-sided *p* value less than 0.05 was regarded of statistically significance.

The β-values were computed from the data of DNA methylation BeadChip and the signal of the final hybridization products. The average β value indicates the methylation levels for each given loci, ranging from 0% to 100%. After the methylation assay, the data were processed in the following order: background correction, probe scaling, quantile normalization, and logit transformation. We performed differential methylation analysis based on logit-transformed values and conducted the Wilcoxon rank test to compare 5 AR samples to 5 NAR samples. The Benjamini–Hochberg method was used to correct *p*-values by calculating the false discovery rate. Probes with adjusted *p*-values <0.01 and delta β-values of 0.2 or −0.2 were considered differentially methylated and statistically significant.

GraphPad Prism 7 was used to assemble the figure panels. BeadStudio Methylation Module v3.3 software (Illumina, Inc.) (http://www.illumina.com) was applied to select and analyze DML. PyroMark Q24 software (Qiagen) was performed to analyze the pyrosequencing data. MultiExperiment Viewer was used to carried out the clustering analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp/; a bioinformatics database) was employed to select differentially methylated genes. The GO (http://geneontology.org/) and KEGG (http://www.genome.jp/kegg/) databases were chosen for functional and pathway enrichment analysis.

3 | RESULTS

3.1 | The characteristics of AR and NAR patients included in the microarray analysis

From the peripheral blood samples of five AR and five NAR controls, the total DNA was extracted for the blood genomic DNA methylation assay. The baseline of these 10 patients (5 AR and 5 NAR) are presented in Table 1. All of the clinical characteristics are well-matched, and no significant differences are discovered between these cases and controls.

3.2 | Global changes in DNA methylation profile of AR

By Genome Studio V2011 software, our study revealed the β-values of 853,307 DNA methylation sites in patients with AR ($n = 5$) and NAR ($n = 5$) (Figure 1). After statistical analysis, it was reported that 7707 CpG sites showed a difference in DNA methylation level. A total of 270 sites were hypermethylated, and 7437

Index	AR ($n = 5$)	NAR ($n = 5$)	t	p Value
Gender (male/female)	2/3	3/2	—	—
Age (years)	66.4 ± 9.9	63.2 ± 8.8	−0.539	0.604
BMI (kg/m^2)	21.53 ± 4.04	22.36 ± 3.22	0.364	0.725
Total cholesterol (mmol/L)	4.19 ± 0.87	5.03 ± 0.87	1.540	0.162
Triglyceride (mmol/L)	2.334 ± 1.808	2.802 ± 2.200	0.368	0.732
LDL-c (mmol/L)	2.728 ± 0.972	3.038 ± 0.557	0.619	0.553
HbA1c (%)	6.68 ± 1.51	6.98 ± 1.69	0.299	0.772
LVEF (%)	63.6 ± 11.8	67.6 ± 5.6	0.683	0.514
Blood sugar	5.900 ± 0.806	7.677 ± 2.347	0.987	0.397
ALT (U/L)	35.0 ± 24.1	44.0 ± 21.9	0.619	0.533
AST (U/L)	64.2 ± 61.6	65.2 ± 32.6	0.032	0.975
Cr (μmol/L)	57.54 ± 33.41	54.50 ± 13.71	−0.188	0.855
Albumin (g/L)	34.90 ± 5.92	36.72 ± 6.81	0.451	0.664
MPV (fl)	8.58 ± 2.93	8.34 ± 1.81	−0.156	0.880
PDW (fl)	16.98 ± 1.07	16.76 ± 0.98	−0.338	0.744
BUN (mmol/L)	6.608 ± 2.945	6.598 ± 0.398	−0.008	0.994
Uric acid (ummol/L)	330.2 ± 147.4	296.8 ± 115.2	−0.399	0.700
HsCRP (mg/dl)	12.612 ± 18.404	14.610 ± 9.504	0.216	0.835
PLT (10^9/L)	189.2 ± 670.2	206.4 ± 32.8	0.496	0.633
PCT	0.16 ± 0.05	0.17 ± 0.02	0.174	0.866

ALT, glutamate aminotransferase; AST, glutamic oxaloacetic transaminase; BUN, blood urea nitrogen; Cr, creatinine; HsCRP, high sensitivity C-reactive protein; LDL-c, low density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; MPV, mean platelet volume; PCT, platelet hematocrit; PDW, platelet distribution width; PLT, platelet.

sites were hypomethylated. Next, we plotted the distribution of differentially hypomethylated and hypermethylated locus across each chromosomes (Table 2). On the basis of the above results, the most hypomethylated locus were located on chromosomes 1, 19 and 17, the most hypermethylated locus on chromosomes 1, 2 and 6.

Then, our group implemented an analysis in accordance with the functional domains of DNA. Among the hypermethylated sites, 82 sites were located in gene bodies (30.3%), 70 sites in noncoding intergenic domains (25.9%) and 49 (18.1%) locus situated within 1500 bp upstream of a TSS (Figure 2A). For the hypomethylated locus, there were 3193 situated in gene bodies (42.5%), 2309 sites in noncoding intergenic domains (31.0%), as well as 782 sites situated within 1500 bp upstream of a TSS (10.5%) (Figure 2A). Mostly hypermethylated and hypomethylated locus both lie in the body regions of genes. What was more, the lowest percentage (0.3%) of hypermethylated loci were located in ExonBnd regions, as were the lowest percentage (0.9%) of mostly hypomethylated loci (Figure 2A). Among the 7707 DMS, 1745 sites were situated in promoter regions, and the ratio (114/270 = 0.42) of hypermethylated sites was higher than that (1631/7437 = 0.22) of hypomethylated sites in the whole genome.

The regional distribution of the differentially methylated CpG loci was estimated according to their distance from the nearest CpG island. Shore and shelf regions were located 0–2 kb and 2–4 kb away from CpG islands, respectively, and open sea regions were isolated loci. Since the AR cases compared to the control group, our group identified that most hypomethylated were suited in N-shore regions (28.7%), while most hypermethylated sites (52.2%) were lied in CpG islands, (Figure 2B). The smallest percentage (12.8%) of hypomethylated loci were situated in islands, whereas hypermethylated sites (2.6%) were discovered in N-shelf regions (Figure 2B).

3.3 | Revealing functional genes from differentially methylated DNA sites

The differentially methylated loci were analyzed on basis of known functional genes with DAVID bioinformatics database. As displayed in Table 3, among the 7707 differentially loci, the top 10 genes with the greatest extent of hypermethylation were APOC2, PMM2, SH3BP5, LINC00094, APOC2, HRH1, FAM136A, ZNF181, OTOA, and CGRRF1. Five of the above 10 genes' relevant

FIGURE 1 Hierarchical clustering heatmap of differentially methylated DNA sites in aspirin resistance (n = 5) and NAR (n = 5) patients.
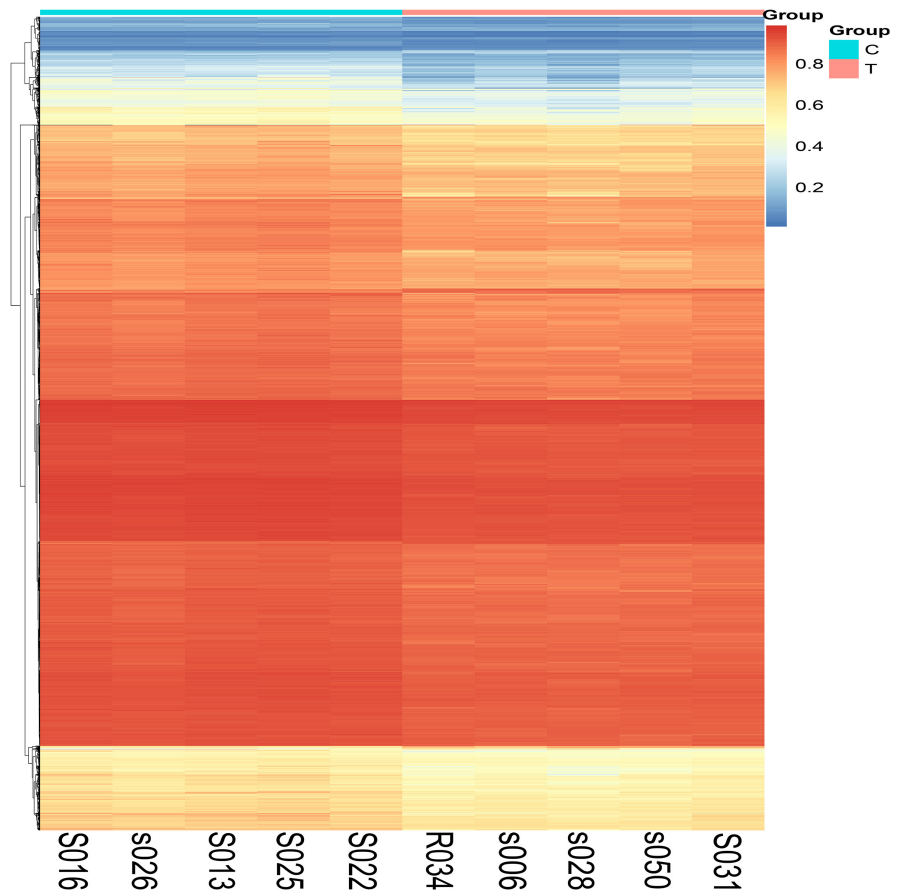
differentially loci of DNA methylation were located around gene body. A subgroup of either the hypermethylated or hypomethylated genes consisted of SNPs in the query sites. The top 10 genes with the greatest extent of hypomethylation were SLC12A5, APOC4, ATXN10, LRTM2, KCNV1, CCR3, RAX, TOP1P2, C7orf16, and ADARB2. Nine of these 10 genes were associated with SNPs, while seven loci were situated in promoter regions and three in the gene body.

3.4 | GO enrichment analysis of differentially methylated genes in AR patients

In the context of biological processing systems (BP pathways), 7707 differentially methylated loci were enriched in 10 groups, including (1) single-organism developmental process (GO:0044767); (2) developmental process (GO:0032502); (3) anatomical structure development (GO:0048856); (4) cell projection organization (GO:0030030); (5) nervous system development (GO:0007399); (6) system development (GO:0048731); (7) multicellular organismal development (GO:0007275); (8) anatomical structure formation involved in morphogenesis (GO:0048646); (9) phosphate-containing compound metabolic process (GO:0006796); and (10) phosphorus metabolic process (GO:0006793). The extent of methylation changes showed significant differences between the AR and NAR for each BP category above and fold enrichment of these pathways were ranged from 1.2 to 1.5 (Figure 3).

In the context of cellular component (CC) pathways, through bioinformatic profiling via the GO and DAVID databases, the genes connected to differentially methylated sites could be arranged into 10 groups: (1) cell projection (GO:0042995); (2) cytoplasm (GO:0005737); (3) cell junction (GO:0030054); (4) neuron part (GO:0097458); (5) neuron projection (GO:0043005); (6) cell leading edge (GO:0031252); (7) cell projection part (GO:0044463); (8) lamellipodium (GO:0030027); (9) plasma membrane part (GO:0044459); and (10) cell periphery (GO:0071944). Statistical analyses interpreted that the extent of methylation presented significant differences in CC categories between AR and NAR, with the fold enrichment ranging from 1.1 to 2.0 (Figure 3).

Under the category of molecular functions, the differentially methylated genes were arranged into following 10 groups: (1) purine nucleotide binding (GO:0017076); (2) purine ribonucleotide binding (GO:0032555); (3) ribonucleoside binding (GO:0032549); (4) purine ribonucleoside binding (GO:0032550); (5) nucleoside binding (GO:0001882); (6) purine nucleoside binding (GO:0001883); (7) ion binding (GO:0043167); (8) ribonucleotide binding (GO:0032553); (9) cytoskeletal protein binding (GO:0008092); and (10) phospholipid binding (GO:0005543). The extent of differential methylation indicated significant differences in each category above. The fold enrichment ranged from over 1.1 to nearly 1.6 (Figure 3).

TABLE 2 Frequency of differentially methylated sites by chromosome after normalization according to chromosome length.

Chromosome	Frequency hypo	Frequency hyper	Total frequency
1	21	698	719
2	15	559	574
3	9	398	407
4	11	336	347
5	19	345	364
6	14	478	492
7	11	447	458
8	15	357	372
9	11	215	226
10	13	382	395
11	17	403	420
12	12	412	424
13	12	193	205
14	10	275	285
15	9	243	252
16	13	342	355
17	20	436	456
18	4	128	132
19	21	339	360
20	4	190	194
21	5	84	89
22	4	177	181

3.5 | KEGG enrichment analysis of differentially methylated genes in AR group

After bioinformatics estimation by the KEGG database, the extent of differential methylation showed a significant difference between the AR and NAR. And the genes with differentially methylated sites in the AR group could be organized into genomic networks under the potential biological interactions, and Table 4 listed the top 10 pathways with the fold enrichment ranging from 1.6 to 2.1, such as circadian rhythms (hsa04710) and insulin secretion (hsa05323).

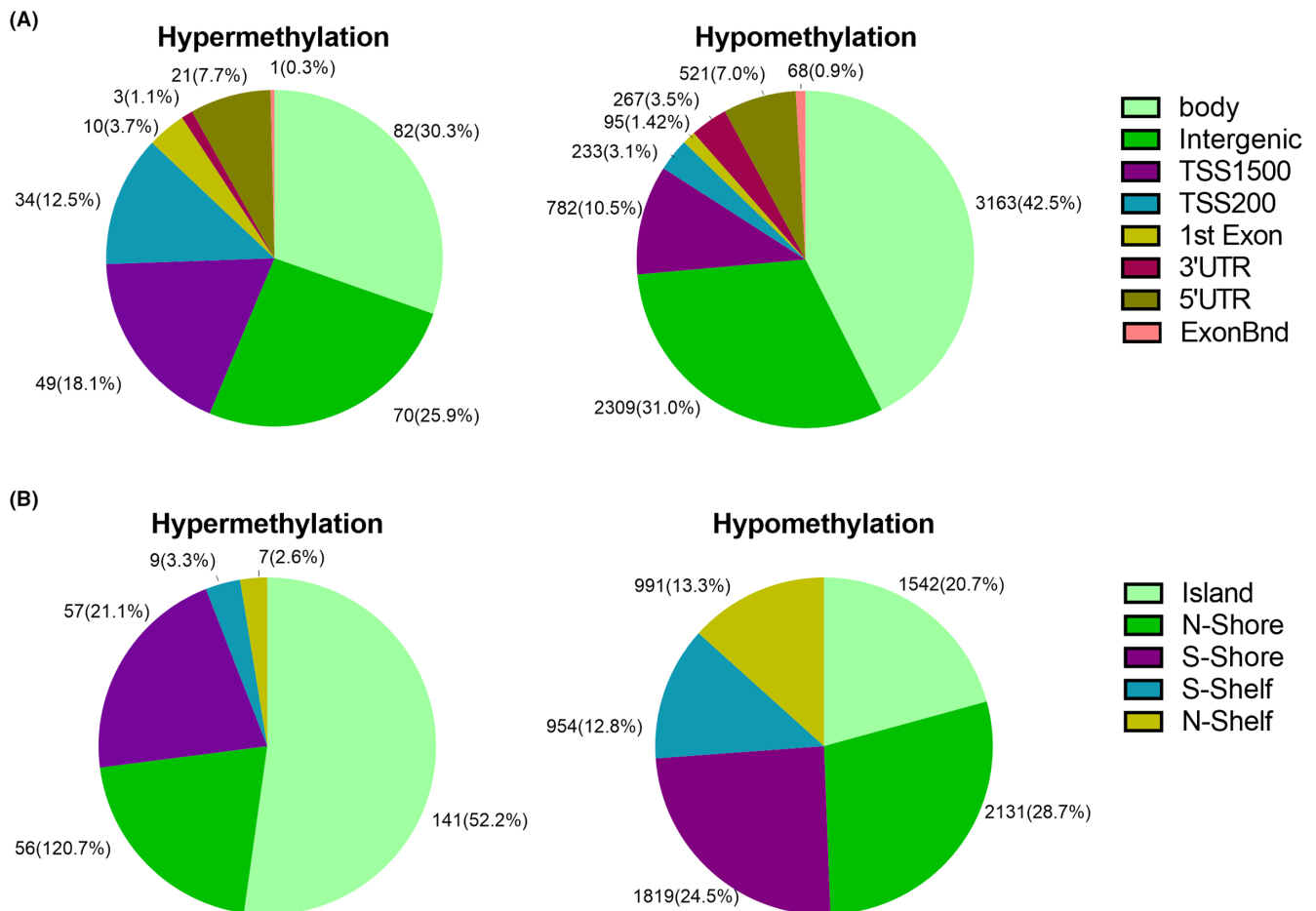## 3.6 | Validation of the differentially methylated CpG loci

Thirty subjects with AR and 30 NAR controls were selected to assess the accuracy of the results from the 850 BeadChip assay of DNA Methylation. As shown in Table 5, the clinical characteristics of these subjects (30 AR vs. 30 NAR) were analyzed. Our study indicated that, compared with NAR, the index of Cr ($p = 0.022$) was significantly higher in the AR group. The other clinical values were shown to have no significant difference ($p > 0.05$).

The results of the pyrosequencing of six sites (cg16391727, cg21463518, cg21008208, cg21293749, cg13945576, and cg13703859) were as followings. For cg16391727, the level of DNA methylation were $23.27 \pm 0.74\%$ in the AR group and $25.66 \pm 0.83\%$ in the control group ($p = 0.036$) (Figure 4A). As shown by pyrosequencing, the cg21463518 presented a methylation rate of $25.92 \pm 2.66\%$ in the AR group and $31.51 \pm 2.46\%$ in the NAR group ($p = 0.128$) (Figure 4B). Meanwhile, the pyrosequencing revealed cg21008208 methylation rates of $31.44 \pm 0.97\%$ in the AR group and $34.91 \pm 0.97\%$ in the control group ($p = 0.014$) (Figure 4C). The cg21293749 site had a methylation rate of $35.93 \pm 0.997\%$ in the AR group and $40.34 \pm 1.07\%$ in the control group ($p = 0.003$) (Figure 4D). For cg13945576, the methylation level were $74.54 \pm 1.30\%$ in the AR group and $78.02 \pm 1.08\%$ in NAR group ($p = 0.044$) (Figure 4E). Lastly, the cg13703859 site had a methylation rate of $27.36 \pm 1.39\%$ in the AR group and $27.28 \pm 1.23\%$ in the control group ($p = 0.964$) (Figure 4F).

The above six differentially methylated loci were located in the genes of *PRKAG2*, *ROR1*, *ROR2*, which were the key molecular in the pathway of circadian rhythms. We evaluated the relative mRNA expression of these three genes through qRT-PCR. The results presented that the mRNA expression of *ROR1* ($p = 0.007$) (Figure 5B) was increased in the AR group, and there was no significant difference between AR and NAR regarding the mRNA expression of the *PRKAG2* ($p = 0.131$) (Figure 5A) and *ROR2* genes ($p = 0.079$) (Figure 5C).

4 | DISCUSSION

Methylation modifications are important epigenetic modification that bind covalently to 70% of CpG (cytosine-phosphate-guanine) dinucleotides in mammalian genomes.[33] CpG islands (CGIs) are regions rich in CpG sites in promoter regions. DNA methylation affects the expression of downstream gene without altering their sequence, which is an important characteristics of epigenetic modification. Hypermethylation of CGIs is relevant to the transcriptional silencing of gene expression, while hypomethylation may lead to higher expression.[34] The mechanisms of gene repression include directly inhibiting the transcription factor binding and indirectly inhibiting such as histone deacetylase complexes as well as methylcytosine-binding proteins.[35] And then, the levels of DNA methylation was probably to be significantly transformed in disease conditions.[36] Differential DNA methylation are covered in various diseases, which are consisted of systemic lupus erythematosus,[37] Parkinson's disease,[38] breast cancer,[39] and intestinal disease.[40] What is more, DNA methylation has a significant influence on cardiovascular diseases. Latest epigenetic studies revealed that DNA methylation is interrelated to ACS,[41] incident CAD or myocardial infarction,[42] idiopathic dilated cardiomyopathy,[43] recovery after myocardial infarction,[44] and susceptibility to hypertension.[45,46] Data on epigenetic modification has accumulated rapidly these days, which will further contribute to our knowledge of aspirin resistance.

(A)

Hypermethylation

21(7.7%) 1(0.3%)
3(1.1%)
10(3.7%)
34(12.5%)
82(30.3%)
49(18.1%)
70(25.9%)

Hypomethylation

521(7.0%) 68(0.9%)
267(3.5%)
95(1.42%)
233(3.1%)
782(10.5%)
3163(42.5%)
2309(31.0%)

- body
- Intergenic
- TSS1500
- TSS200
- 1st Exon
- 3'UTR
- 5'UTR
- ExonBnd

(B)

Hypermethylation

9(3.3%) 7(2.6%)
57(21.1%)
141(52.2%)
56(120.7%)

Hypomethylation

991(13.3%) 1542(20.7%)
954(12.8%)
2131(28.7%)
1819(24.5%)

- Island
- N-Shore
- S-Shore
- S-Shelf
- N-Shelf

FIGURE 2 (A) Frequency of differentially methylated regions according to their functional distribution. (B) Frequency of differentially methylated sites according to their distribution in the CpG island context.

Platelets can be activated through three pathways resulting in platelet aggregation: the thromboxane A2 (TXA2), adenosine diphosphate (ADP)-P2Y12 receptor and glycoprotein (GP) IIb/IIIa receptor pathways. Aspirin, also known as acetylsalicylic acid, is an antiplatelet agent that is routinely recommended for the basic prevention of adverse ischemic events. The main pharmacological mechanism of aspirin is the irreversible acetylation induction of the cyclooxygenase (COX)-1 enzyme and the inhibition of thromboxane A2 (TXA2) and prostacyclin (PGI2) biosynthesis from arachidonic acid (AA), ultimately blocking platelet activation and aggregation.[47] As mentioned above, genetic studies on the therapeutic targets of aspirin based on this pharmacological process have revealed new possibilities for addressing AR. One notable research direction is related to the P1[A] polymorphism in GP IIIa, which leads to the substitution of leucine by proline in 33rd subunit of GP IIIa, leading to an increase in platelet activity under aspirin therapy, such that larger doses of aspirin are required for allele carriers to achieve the same antiplatelet effect as homozygotes.[48] This proves that genetic research can provide accurate recommendations for clinical aspirin therapy, as can the examination of gene methylation in epigenetic studies. It has been confirmed that promoter methylation of the ABCB1 gene can affect the translation and posttranscriptional modification of

P-glycoprotein, which reduces the intestinal absorption of aspirin and leads to AR.[49] Some data have indicated that gene methylation may be responsible for aspirin resistance, although there are limited studies on the effect of gene methylation on AR in ACS patients. The current study of whole-genome DNA methylation may contribute to the study of AR, potentially revealing relevant biomarkers and underlying pathways.

In this study, microarray assay theoretically identifies methylation changes across the whole genome. Our results identified 7707 differentially methylated sites, including 270 hypermethylated positions and 7437 hypomethylated positions in the AR subjects compared with the NAR group. The ratio (hypermethylated/hypomethylated sites) was 0.036. We found that lower methylation occurred at more locus than higher methylation, based on the results showing that the ratio of hypermethylated to hypomethylated locus in promoter regions was 0.42 (114/270). During data analysis, the DNA methylation alterations found in the AR groups were located in genes that were not closely connected with aspirin pharmacokinetics. The bioinformatic profile suggested that the differentially methylated locus might be linked to several functional genes. Eight functional genes (*PMM2, SH3BP5, FAM136A, OTOA, CGRRF1, SLC12A5, ATXN10 and ADARB2*) among the top 20 significantly differentially methylated

TABLE 3 Top 10 hypermethylated and hypomethylated genes in the whole-blood genome in CR.

	Target ID	Gene ID	delta β	p Value	CHR	Location	SNP
Top 10 hypermethylated genes	cg09555818	APOC2	0.124579974	0.00011774	19	1stExon	rs183066391; rs2288911; rs561256642
	cg01866455	PMM2	0.088408645	0.000133539	16	Body	rs368793160; rs555699961; rs140486173; rs556509621
	cg24170085	SH3BP5	0.053123245	0.000144384	3	Body	rs142686824; rs144294550
	cg19852650	LINC00094	0.088523132	0.00015564	9	TSS1500	rs149044655; rs109536
	cg13119609	APOC2	0.118356987	0.000315597	19	1stExon	rs183066391; rs2288911; rs561256642
	cg01331196	HRH1	0.204084286	0.000333082	3	5'UTR	
	cg22976360	FAM136A	0.053143684	0.000336347	2	Body	
	cg15085454	ZNF181	0.096924353	0.000521145	19	TSS200	rs113836067; rs560559184
	cg05909984	OTOA	0.126430989	0.000824498	16	Body	rs12599397; rs559540158
	cg18492804	CGRRF1	0.032004145	0.000833224	14	Body	rs549382141; rs567950286
Top 10 hypomethylated genes	cg26828689	SLC12A5	−0.223793377	1.01395E-05	20	Body	rs191955711; rs549581842
	cg11985722	APOC4	−0.048069895	1.84333E-05	19	TSS1500	rs143895802; rs117812557
	cg09049978	ATXN10	−0.0561616	2.65701E-05	22	Body	rs182400120; rs186678549; rs534834694
	cg25629418	LRTM2	−0.058167725	2.66366E-05	12	TSS200;	rs185249002
	cg21225797	KCNV1	−0.075169413	3.42332E-05	8	TSS1500	rs560331953; rs527666433; rs543379565; rs561585339
	cg04111761	CCR3	−0.065364831	3.45572E-05	3	TSS1500	
	cg06620569	RAX	−0.128965886	3.61923E-05	18	TSS1500	rs528113744; rs546876911; rs562021629; rs529223099
	cg03043822	TOP1P2	−0.131175326	3.91864E-05	22	TSS1500	
	cg01353448	C7orf16	−0.140463776	4.50303E-05	7	5'UTR	rs142366665; rs537834940
	cg08227227	ADARB2	−0.169468737	4.75728E-05	10	Body	rs75471900; rs2813410

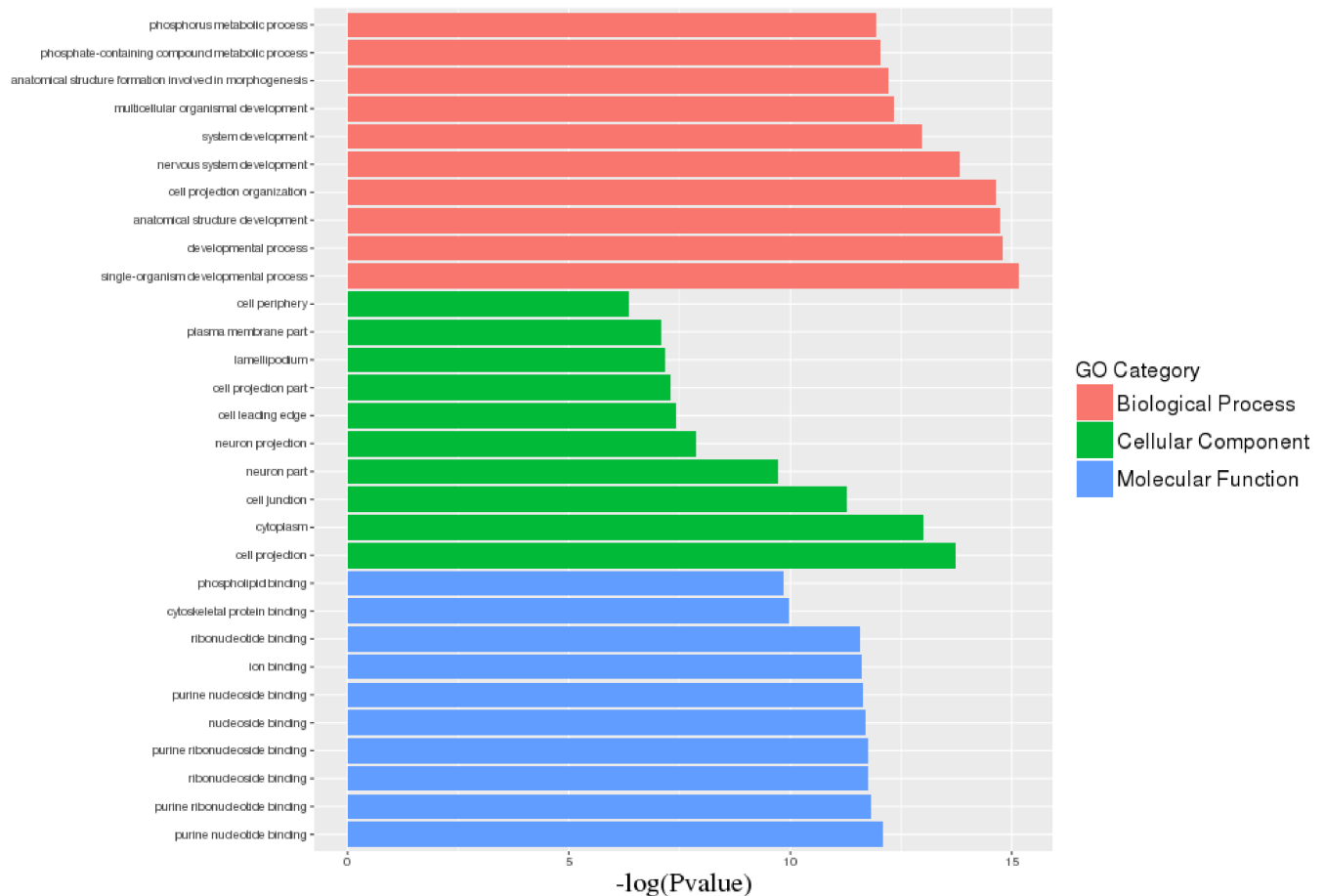Abbreviations: CHR, chromosome; SNP, single nucleotide polymorphism.

FIGURE 3 GO functional annotation of differentially methylated genes in patients with aspirin resistance.

genes (DEGs) were associated with AR (Table 5). The above finding confirms that multiple genetic or epigenetic factors are interrelated to complex human diseases which were consisted of AR, by participating in various cellular processes. DEGs have the potential to serve as predictors. Through pyrosequencing and qRT-PCR validation, our group confirmed that the cg16391727, cg21008208, cg21293749, and cg13945576 were associated with the risk of AR, which showing no difference with the BeadChip analysis results. cg16391727 and cg21008208 map to PRKAG2, which was encoding the gamma 2 regulatory subunit of adenosine monophosphate-activated protein kinase (AMPK). CpG cg21293749 maps to the ROR1 (retinoic acid receptor-related orphan receptor 1), and CpG cg13945576 maps to the ROR2 (retinoic acid receptor-related orphan receptor 2). ROR is one of the six key genes (*CLOCK, BMAL, PER, CRY, REV-ERB, ROR*) responsible for circadian rhythms. ROR1 is a transmembrane receptor that contains extracellular, transmembrane, as well as cytoplasmic domains. The extracellular portion includes kringle, cysteine-rich, and immunoglobulin-like domains. Sibbing et al. showed that platelet activity presented circadian rhythm characteristics,[50] which provides clues to link *ROR1* to AR. The cg21293749 hypermethylation may inhibit *ROR1* expression, thereby affecting platelet circadian rhythm under aspirin therapy and ultimately leading to AR. Considering these results combined with the qRT-PCR verification

results showing that the mRNA expression of *ROR1* was increased in the AR group, we can infer that the hypomethylation of CpG cg21293749 may alter the expression of *ROR1*, thereby affecting platelet circadian rhythm under aspirin therapy and ultimately leading to AR.

In the light of KEGG analysis, as shown in Table 4, 15 top enrichment pathways, such as the circadian rhythm and insulin secretion signal pathways, are involved. Among them, circadian entrainment is associated with AR. It has been reported that platelet functions have circadian fluctuations and are affected by circadian CLOCK proteins in mouse studies,[51] and mutations in the *CLOCK* gene might lead to platelet hyperaggregability. Similarly, according to our results, methylation of the *ROR1 gene* may affect platelet circadian rhythm, which is a valuable direction in the mechanistic study of AR.

In addition, epigenetics have provided new horizons in biomedicine. In contrast to the classical genetic model, epigenetics refers to heritable changes in phenotype resulting from the changes of gene expression without alteration in the underlying DNA sequence. Epigenetics includes DNA methylation, histone modification, and the RNA landscape. In addition, epigenetics have provided new horizons in biomedicine. Evidence has indicated that epigenetic-related diseases mainly include cardiovascular disease and cancer, providing new potential treatment strategies. Studies have raised the

TABLE 4 The top 15 genomic networks with altered methylation in aspirin resistance based on KEGG analysis.

Pathway_id	Pathway_name	DMS involved	% total DMS	Genes	Fold enrichment	p Value	FDR
hsa04724	Glutamatergic synapse	7	6.60%	SHANK2, CACNA1C, GRIN2B, PRKACA, SLC1A6, GRIK5, GNAS	3.59E+00	2.95E-03	2.07E-01
hsa05120	Epithelial cell signaling in *Helicobacter pylori* infection	5	4.72%	TCIRG1, ATP6V0A2, SRC, NOD1, LYN	4.44E+00	4.97E-03	2.07E-01
hsa04728	Dopaminergic synapse	7	6.60%	CACNA1C, GRIN2B, AKT1, CREB3L3, PRKACA, GNAS, COMT	3.25E+00	5.04E-03	2.07E-01
hsa05031	Amphetamine addiction	5	4.72%	CACNA1C, GRIN2B, CREB3L3, PRKACA, GNAS	4.37E+00	5.29E-03	2.07E-01
hsa04962	Vasopressin-regulated water reabsorption	4	3.77%	CREB3L3, PRKACA, DCTN1, GNAS	5.28E+00	6.49E-03	2.07E-01
hsa04976	Bile secretion	5	4.72%	FXYD2, PRKACA, ABCG5, ABCG8, GNAS	4.13E+00	6.72E-03	2.07E-01
hsa05030	Cocaine addiction	4	3.77%	GRIN2B, CREB3L3, PRKACA, GNAS	4.76E+00	9.39E-03	2.07E-01
hsa05203	Viral carcinogenesis	8	7.55%	TP53, CREB3L3, PRKACA, HDAC4, SRC, GTF2H4, LYN, SCRIB	2.52E+00	1.19E-02	2.07E-01
hsa05216	Thyroid cancer	3	2.83%	TP53, PAX8, RXRB	6.15E+00	1.22E-02	2.07E-01
hsa05110	Vibrio cholerae infection	4	3.77%	TCIRG1, ATP6V0A2, PRKACA, GNAS	4.40E+00	1.22E-02	2.07E-01
hsa04710	Circadian rhythm	3	2.83%	CRY2, RORA, PRKAG2	5.95E+00	1.34E-02	2.07E-01
hsa04911	Insulin secretion	5	4.72%	FXYD2, CACNA1C, CREB3L3, PRKACA, GNAS	3.46E+00	1.38E-02	2.07E-01
hsa05323	Rheumatoid arthritis	5	4.72%	TCIRG1, ATP6V0A2, ACP5, HLA-DOA, HLA-DPB1	3.42E+00	1.45E-02	2.07E-01
hsa00740	Riboflavin metabolism	2	1.89%	FLAD1, ACP5	9.91E+00	1.65E-02	2.20E-01
hsa05202	Transcriptional misregulation in cancer	7	6.60%	PBX1, SLC45A3, IGF1R, TP53, PAX8, RXRB, PAX5	2.52E+00	1.84E-02	2.28E-01

novel possibility that human heart failure may be treated by histone deacetylase inhibition.[52] Epigenetic mechanisms, especially those related to noncoding RNA and DNA methylation, have recently been intensely explored in the context of aspirin resistance. In regard to noncoding RNAs, many studies have shown a relationship between AR and noncoding RNAs. Lower miR-19b-1-5p expression is associated with AR,[53] and another study suggested that lncRNA H19 would induce AR via ascending the production of 8-iso-PGF2α.[54] What was more, the differential expression of miR-223, miR-191, miR-126, along with miR-150[55] in platelets may be one of the determinants of variability in platelet activity after aspirin use. In terms of gene methylation, ABCB1 hypomethylation is associated with AR through lower drug absorption.[56] At present, the relationship between AR and the methylation of individual genes with specific functions is still unclear and is worthy of further exploration. In terms of histone modification, there are scarce available data associated with AR. Notably, N6-methyladenosine (m6A) has been a hotpot of epigenetic methylation study in recent years. m6A, which is involved in post-transcriptional modification,[57] is considered to play pivotal roles in

RNA cleavage, translation, and expression. This research further elucidated the epigenetic features of AR, revealing more related genes and pathways. On this basis, it can be predicted that the potential effect of m6A may be a new research direction in the study of AR.

This study revealed the characteristics of DNA methylations in AR patients, which is important and innovative work. However, there are still some limitation need to be addressed. Firstly, the study are lack of basal ARU detection before aspirin treatment. The limited sample size and the lack of large-scale and multiregional sampling and analysis are additional limitations of this work. Finally, based on cellular or animal experiments, advanced mechanistic validation needs to be implemented.

In conclusion, this was the first dataset from humans with AR indicating DMGs in the whole-blood genome and it demonstrated that differential methylation changes in cg16391727, cg21008208, cg21293749, and cg13945576 are related to AR. The *ROR1* mRNA expression was increasing in AR patients, suggesting that an alteration in the DNA methylation of cg21293749 may lead to differential expression of *ROR1*, ultimately resulting in AR. Furthermore, the

TABLE 5 The clinical characteristics of aspirin resistance (AR) and non-aspirin resistance (NAR) patients in the validation cohort

Index	NAR (n = 30)	AR (n = 30)	z/t/χ^2	p Value
HsCRP (mg/dl)	2.000 (0.993, 8.498)	3.105 (0.875, 5.685)	−0.081	0.935
AST (μmol/L)	27.0 (18.0, 78.3)	23.0 (16.8, 53.0)	−0.940	0.347
HbA1c (%)	6.37±1.24	6.28±1.19	0.287	0.775
Triglyceride (mg/dl)	1.718±1.132	1.602±0.918	0.435	0.665
Blood sugar	5.505±1.272	5.399±1.428	0.305	0.762
BUN (mmol/L)	5.645±1.980	5.798±2.011	−0.298	0.767
Age (years)	60.7±8.6	64.5±8.5	−1.740	0.087
Left ventricular ejection fraction (%)	61.1±6.8	58.5±8.5	0.322	0.201
ALT (μmol/L)	40.8±35.0	32.8±27.0	0.996	0.323
Uric acid (μmmol/L)	327.4±97.3	339.9±125.9	−0.431	0.668
PLT (10^9/L)	196.8±48.9	190.7±73.2	0.380	0.706
TBIL (mmol/L)	14.34±6.65	13.37±9.00	0.478	0.635
Albumin (g/L)	40.06±4.58	38.67±3.13	1.375	0.174
Cr (μmol/L)	69.44±17.36	80.15±17.91	−2.352	0.022
MPV (fl)	8.53±1.29	8.13±1.30	1.176	0.244
BMI (kg/m^2)	24.204±3.149	23.866±2.402	0.467	0.642
Total cholesterol (mg/dl)	4.759±1.231	4.287±1.075	1.547	0.127
HDL (mg/dl)	1.006±0.240	0.986±0.354	0.248	0.805
LDL (mg/dl)	2.896±0.990	2.532±0.932	1.466	0.148
PCT (%)	0.166±0.036	0.159±0.043	−2.048	0.499
Male gender, n (%)	23 (76.7)	21 (70.0)	0.341	0.559
Alcohol abuse, n (%)	6 (20.0)	7 (23.3)	0.098	0.754
Hypertension, n (%)	20 (66.7)	21 (70.0)	0.077	0.781
Diabetes, n (%)	7 (23.3)	8 (26.7)	0.089	0.766
Dyslipidemia, n (%)	12 (53.3)	11 (60.0)	0.071	0.791
Current smoking, n (%)	16 (53.3)	12 (40.0)	1.071	0.301

FIGURE 4 Cross-validation of DNA methylation with pyrosequencing. The mean methylation: (A) cg16391727, AR vs. NAR: 23.27±0.74% vs. 25.66±0.83%, p = 0.036. (B) cg21463518, AR vs. NAR: 25.92±2.66% vs. 31.51±2.46%, p = 0.128. (C) cg21008208, AR vs. NAR: 31.44±0.97% vs. 34.91±0.97%, p = 0.014. (D) cg21293749, AR vs. NAR: 35.93±0.997% vs. 40.34±1.07%, p = 0.003. (E) cg13945576, AR vs. NAR: 74.54±1.30% vs. 78.02±1.08%, p = 0.044. (F) cg13703859, AR vs. NAR: 27.36±1.39% vs. 27.28±1.23%, p = 0.964. AR, aspirin resistance; NAR, non-aspirin resistance.
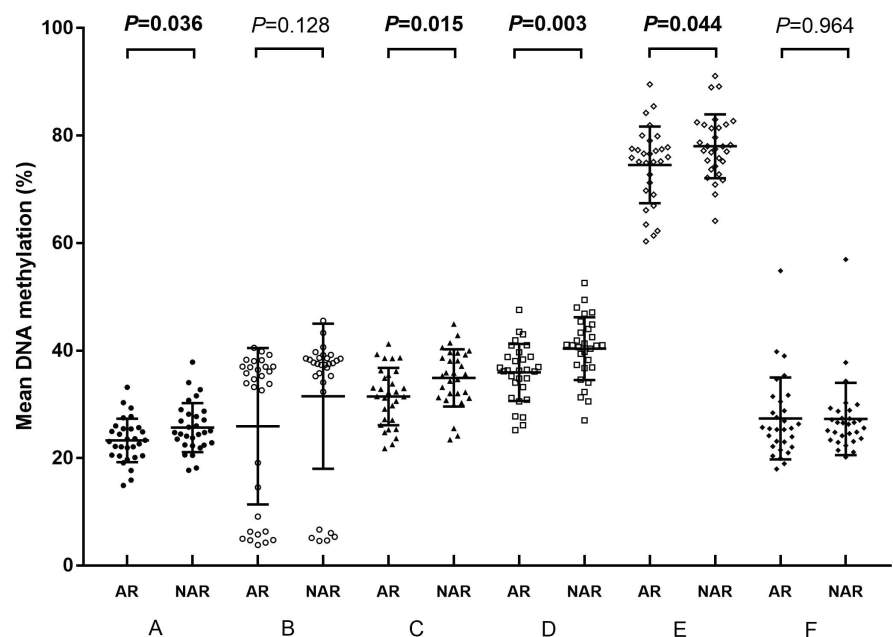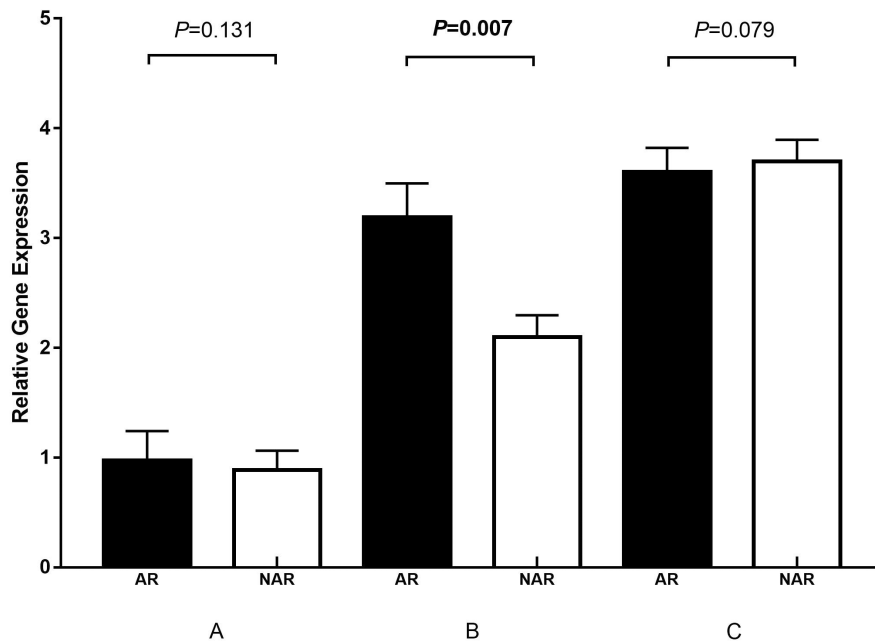
FIGURE 5 A comparison of the mRNA expression levels of three genes (PRKAG2, ROR1, and ROR2) between AR and NAR. (A) PRKAG2, AR vs. NAR: 0.976±0.046 vs. 0.886±0.032, p = 0.131. (B) ROR1, AR vs. NAR: 3.190±0.057 vs. 2.101±0.037, p = 0.007. (C) ROR2, AR vs. NAR: 3.601±0.040 vs. 3.698±0.036, p = 0.079. AR, aspirin resistance; NAR, non-aspirin resistance.

identified DMGs are related to molecular pathways and biomarkers, which are findings of profound value for early clinical evaluation. The genetic and molecular mechanisms of AR are still under exploration, and the sample size needs to be expanded in multicenter studies with more comprehensive plans and empirical approaches in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.