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RESEARCH ARTICLE

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Hypomethylation of the *opioid receptor delta 1* gene combined with high opioid receptor delta 1 protein levels indicates increased risk of gout

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Abstract

Background: The purpose of this study was to identify biomarkers for the diagnosis of gout in Chinese Han males using methylation microarray profiling.

Methods: We screened for differentially methylated genes (DMGs) in gout using a methylation microarray and analyzed the functions of the DMGs using gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. We verified gene methylation levels by pyrosequencing and protein levels by enzyme-linked immunosorbent assays (ELISAs). Statistical analyses were performed using SPSS. Two-sided p values <0.05 were deemed to be statistically significant for all analyses.

Results: We identified 20,426 significant differential methylation sites (5719 high-methylation sites and 14,707 low-methylation sites). Bioinformatics analysis showed that the DMGs were mainly involved in 43 biological functions, 13 cellular components, 18 molecular functions, and 35 KEGG pathways. We selected *opioid receptor delta 1* (*OPRD1*) for verification of methylation levels between 50 gout patients and 50 controls. The methylation levels of *OPRD1* (Chr1:29,139,121) were significantly lower in the gout group ($p<0.05$), while OPRD1 protein levels were significantly higher in the gout group ($p<0.05$). In addition, the AUC of the combination of *OPRD1* (Chr1:29,139,121) methylation and OPRD1 protein levels was 0.796 (0.710, 0.883) with a high sensitivity of 82% and a specificity of 68% ($p<0.001$).

Conclusions: The combination of *OPRD1* (Chr1:29,139,121) hypomethylation and high levels of OPRD1 protein is a potential biomarker for gout diagnosis.

KEYWORDS
gout, methylation, methylation microarray, diagnosis, OPRD1

1 | INTRODUCTION

Gout is a disease caused by the deposition of monosodium urate monohydrate in the joints, soft tissues, and various other tissues.[1] The condition is associated with uric acid excretion disorder or abnormal metabolism of purine, resulting in reduced uric acid excretion or excessive production of uric acid, respectively.[2] The cause of gout remains unclear; it is generally believed that both genetic and

environmental factors play roles in the pathogenesis of the condition.[3] Gout often has a degree of familial aggregation, which may be related to polygenic inheritance.[4]

Laboratory tests for the diagnosis of gout mainly rely on serum uric acid levels, but only 22% of patients with hyperuricemia will develop gout within the next 5 years.[5] The gold standard for the diagnosis of gout is that needle-like crystals of urate can be observed in the synovial fluid of the affected joint under a polarized light microscope; however, the invasiveness of the joint puncture procedure makes this difficult to achieve. At present, commonly used auxiliary examinations include dual-energy CT (a special color development of urate crystals can be observed) and joint ultrasound (a double-track sign can be seen).[6] Lee and Song suggest that dual-energy CT has a sensitivity of 0.84 and a specificity of 0.93 for the diagnosis of gout.[7] However, positive results of these tests indicate the formation of urate crystals and do not suggest the risk of gout.

DNA methylation refers to a chemical reaction in which the cytosine of a CpG dinucleotide is methylated to 5-methylcytosine under the action of DNA methyltransferases, with the methyl group provided by S-adenosylmethionine.[8,9] Changes in gene methylation are earlier than changes in protein levels, and have certain predictive significance for the risk of disease. A large number of studies have shown that gene methylation plays an important role in tumors and in the nervous and endocrine systems.[10–12] Zhu et al reported that hypomethylation at the promoter region of *NRBP1* reduces the binding of *TFAP2A* and thus leads to elevated *NRBP1* expression, which might contribute to the development of gout.[13]

The array-based Illumina Infinium Human Methylation 450K BeadChip (Illumina450K) facilitates high-throughput analysis and can rapidly and simultaneously detect the methylation status of genes using a small amount of genomic DNA. The aim of this study was to find biomarkers that indicate the risk of gout by identifying differentially methylated genes (DMGs) using methylation microarray profiling of whole genomes of gout patients and to compare them with those of control participants. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses that randomly selected 1000 genes containing differentially methylated CpG sites were used to explore the relationship between gene methylation and the pathogenesis of gout. The findings were verified in a larger number of samples using pyrosequencing and enzyme-linked immunosorbent assay (ELISA).[14,15]

2 | MATERIALS AND METHODS

2.1 | Sample collection

We recruited three participants with gout and three control participants from the Outpatient Department of Ningbo Hwa Mei Hospital, University of Chinese Academy of Sciences, between January 2022 and February 2022. The mean age of the patients with gout was 36.7 ± 6.4 years and that of the controls was 42 ± 2.7 years; the other personal information and laboratory data were not significantly different between the two groups. A 2-mL sample of fasting peripheral venous blood was collected from each participant and stored at −80°C.

In addition, 50 participants with gout (43.0 ± 12.6 years) and 50 age-matched controls (41.1 ± 11.4 years) were selected at the same hospital between January 2022 and April 2022. A 2-ml sample of fasting peripheral venous blood was collected from each of the above participants. Blood cells and plasma were separated by centrifugation, place into separate tubes, and stored at −80°C.

All males were unrelated Han Chinese and had no rheumatic diseases, metabolic diseases, kidney diseases, cancer, or mental illness. Diagnosis of gout was consistent with the 2015 ACR/EULAR gout classification criteria.[16] The institutional review boards in the Ethical Committees at Ningbo Hwa Mei Hospital, University of Chinese Academy of Sciences, approved this study. Personal information and laboratory data, including age, sex, alanine aminotransferase (ALT), aspartate transaminase (AST), creatinine (CREA), uric acid (UA), glucose (GLU), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride levels (TG), and white blood cell (WBC) counts were recorded by experienced rheumatologists.

2.2 | Methylation profiling

Whole genomic DNA was extracted using a QIAamp DNA kit, and the concentration and purity of DNA were determined. A 500-ng DNA aliquot was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research). After PCR amplification, the methylated DNA from the three participants with gout and three controls was fragmented, precipitated, and re-dissolved before being hybridized with an Infinium Methylation 450k microarray. The BeadChips were scanned with the Illumina BeadArray Reader, which uses a laser to excite a fluorophore in the single-base extension product on the beads. The data from high-resolution images of the light emitted from the fluorophores were analyzed to determine methylation profiles using Illumina's methylation analysis software.

2.3 | Pyrosequencing assay

The methylated DNA of 50 patients with gout and 50 controls, extracted using the methods in section 2.2, was amplified by PCR and sequenced; the PCR and sequencing primers were designed by PyroMark Assay Design 2.0. The forward primer sequence of the reference gene (*opioid receptor delta 1; OPRD1*) was 5′-TTGTTGGGT AAAGTGTTTGTTATGT-3′, the reverse primer sequence was 5′-AA ATTCCTAATCCCCACCCTCACCC-3′, and its sequencing primer sequence was 5′-GGGTAAAGTGTTTGTTATGTT-3′. The PCR products

were sequenced on a PyroMark Q96 ID (Qiagen), and the methylation status of sites was evaluated by Pyro Q-CpG.

2.4 | Serum sample preparation and ELISA

ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.) were used to measure protein levels. Different concentrations of standard (50 μl) were added to standard wells, 10 μl of test sample and 40 μl sample dilution buffer were added to the sample wells, and nothing was added to the blank wells. Then 100 μl of detection antibody labeled with horseradish peroxidase was added to all wells. The wells were sealed with sealing film and the plate was placed in a 37°C water bath for 60 min. The liquid was discarded, and each well was washed five times with washing solution. Then, 50 μl of each substrate A and B was added and incubated at 37°C for 15 min in the dark, 50 μl of stop solution was then added, and the OD value of each well was measured at 450 nm using a microplate reader.

2.5 | Statistical analysis

By scanning the signals of the microarray in grayscale, we measured the relative signal values for the methylated sites. We standardized the signal value using the Illumina control method to extract the methylated site data, such as level value (Avg_Beta), and Detection *p* value. The levels of differential methylation sites from the gout and healthy groups were compared using the *t*-test ($p < 0.05$), |delta beta| >0.01. We clustered the differential CpG sites using R4.0.5 software and performed GO term enrichment and KEGG pathway analyses using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

The data for the gout and control groups were analyzed by SPSS 26.0. Continuous variables were evaluated as means ± standard deviation, and Student's *t*-test was used to assess differences in clinical data. Two-sided *p* values <0.05 were deemed to be statistically significant for all analyses.

3 | RESULTS

3.1 | Identification of 20,426 differentially methylated sites (DMSs) and 10,063 DMGs

The methylation microarray includes 12,332 probes for each sample as quality control items to check parameters such as staining, extension, target removal, hybridization, stringency, and nonspecific binding. The results were consistent with normal results from the manufacturer, and the microarray status was deemed normal (Figure 1).

We observed 20,426 DMSs in 10,063 DMGs between the gout and control groups. Of these DMSs, 5719 were hypermethylated and 14,707 were hypomethylated. The DMSs were widely distributed, ranging from chromosome 1 to chromosome 22 and the sex chromosomes. Among chromosomes 1–22, chromosomes 1 and 2 contained the most DMSs (1859 and 1498, respectively), while chromosomes 21 and 18 contained fewer DMSs (237 and 330, respectively). The X chromosome contained 510 and the Y chromosome contained 16 DMSs (Figure 2).

From the 20,426 CpG sites that were differentially methylated, we randomly selected 5000 for cluster analysis. The gout group had more hypomethylated sites, while the control group had more hypermethylated sites (Figure 2).

3.2 | GO term and pathway analysis of DMGs

We then randomly selected 1000 genes containing differentially methylated CpG sites for GO term enrichment analysis, which showed that the 1000 genes were involved in 43 biological processes (BPs), 13 cell components (CCs), and 18 molecular functions

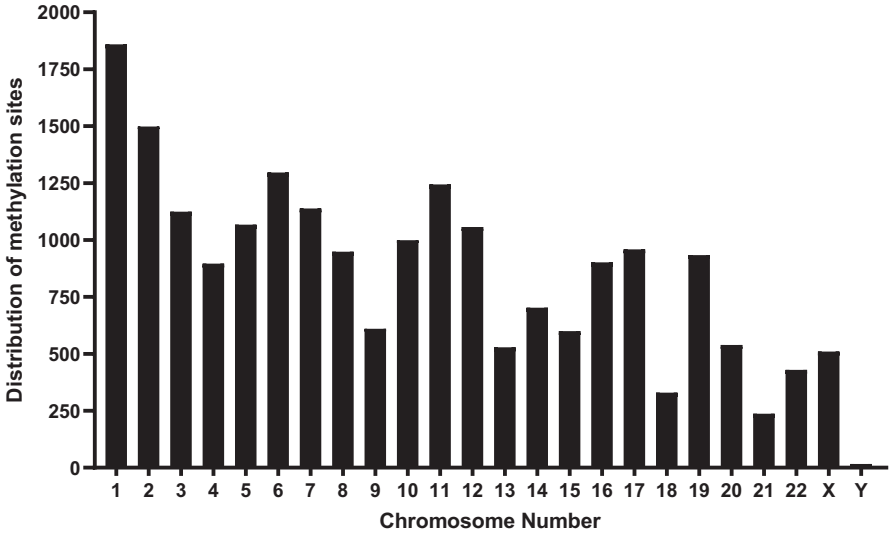

FIGURE 1 Distribution of methylation sites in chromosomes. Among chromosome 1 to chromosome 22, chromosomes 1 and 2 contain the most methylation sites (1859 and 1498, respectively), while chromosomes 21 and 18 contain fewer methylation sites (237 and 330, respectively). Among the sex chromosomes, the X chromosome contains 510 methylation sites and the Y chromosome contains 16 methylation sites.

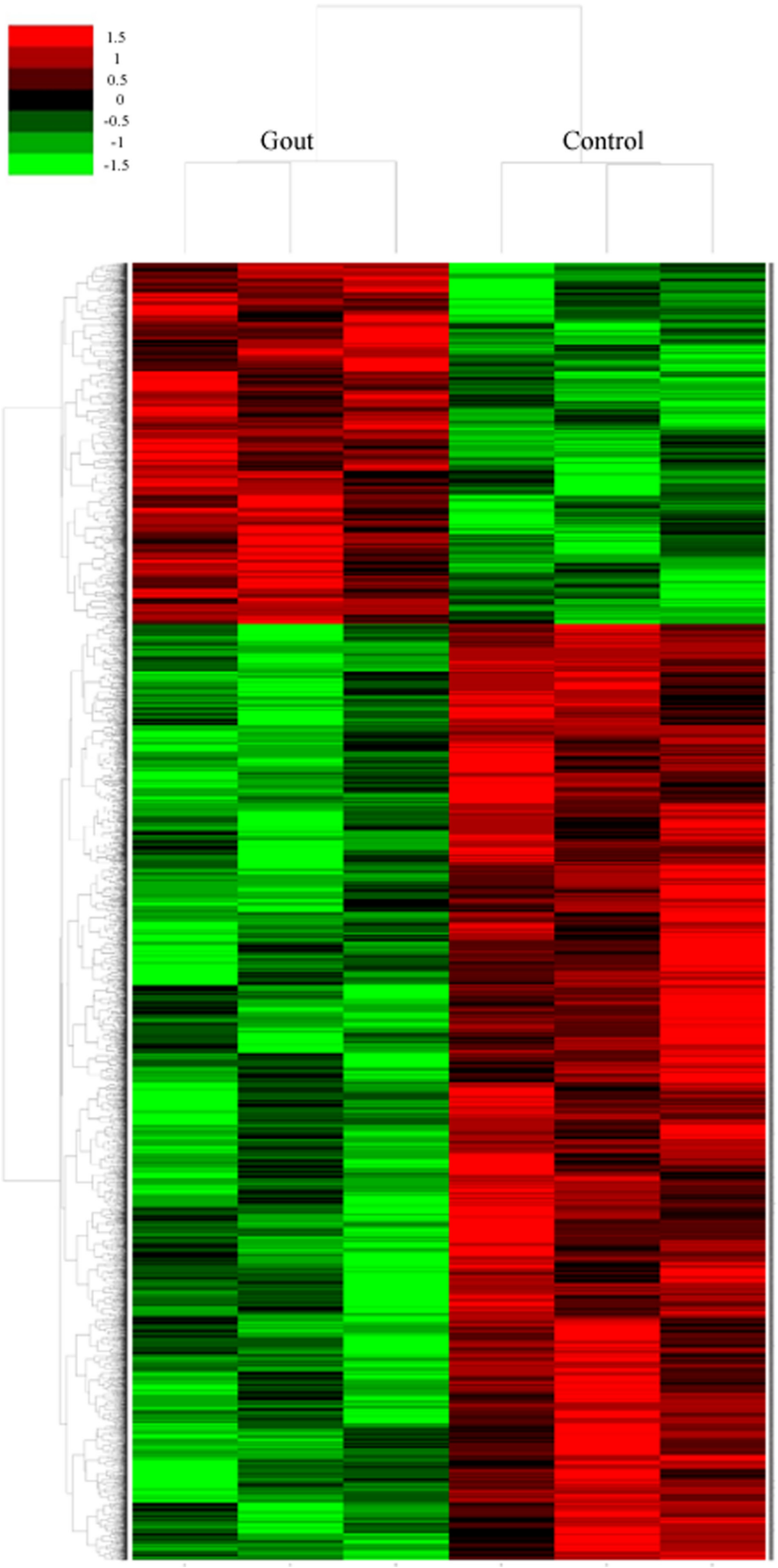

FIGURE 2 Heat map showed that the gout group had more hypomethylated sites, while the control group had more hypermethylated sites

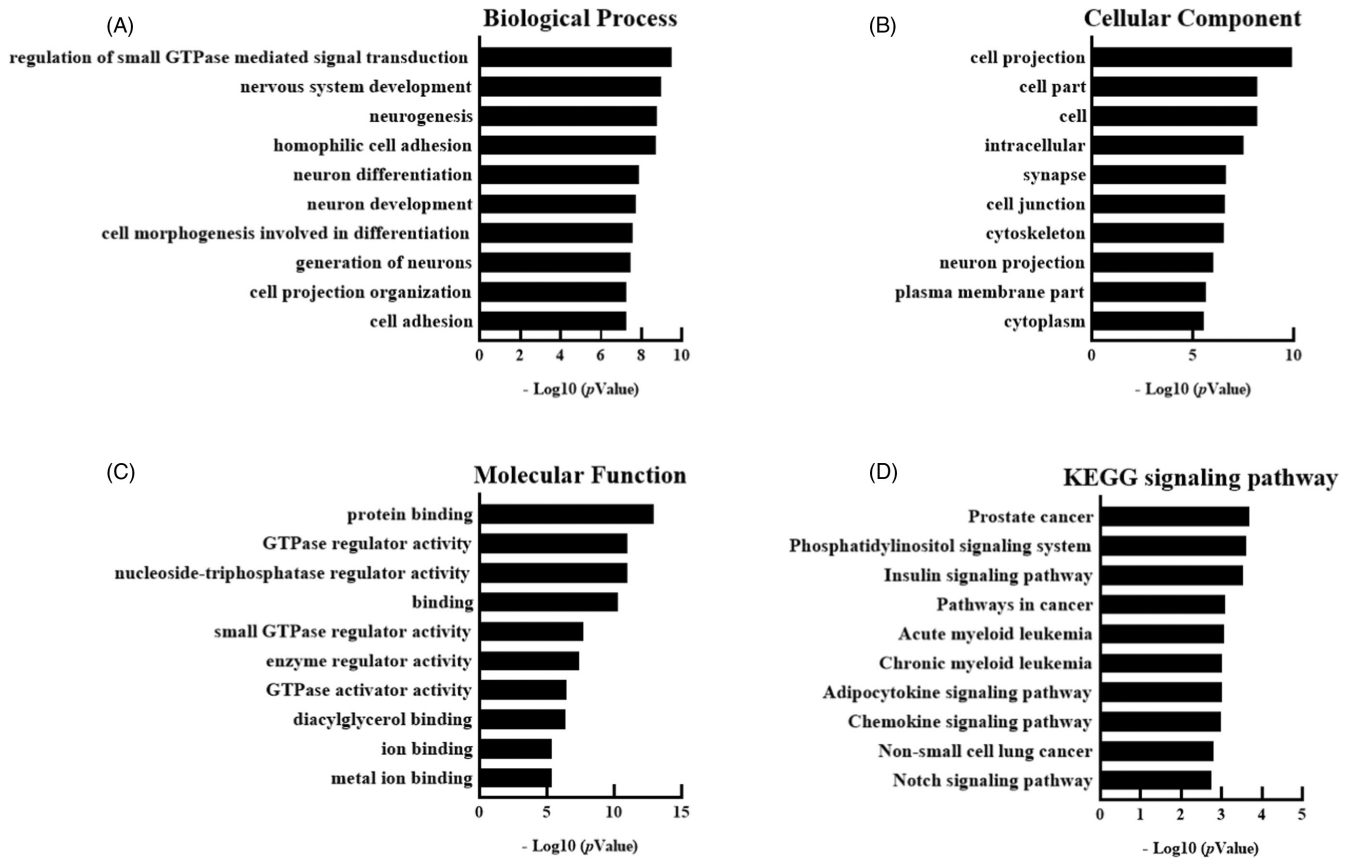

FIGURE 3 Significantly enriched gene ontology histograms and KEGG pathway histogram. It showed the top ten of BPs(A), CCs (B), MFs (C) and KEGG pathways (D). The ordinate represents the significantly enriched gene ontology and KEGG pathway name, and the abscissa represents Log10 of the *p*-value. A larger abscissa indicates that the gene ontology or pathway is more enriched.

(MFs); the results were sorted by *p* value ($p < 0.05$, false discovery rate <0.05) [Supplementary Table S1(A–C)]. The top 10 BPs, CCs, and MFs are listed in Figure 3A–C. The GO functional annotation analysis of the differentially expressed methylation sites showed that these genes were mainly enriched in the BP terms, regulation of small GTPase-mediated signal transduction, nervous system development, neurogenesis, and homophilic cell adhesion. CC analysis showed enrichment for the terms, cell projection, cell part, cell, and intracellular. Analysis of MFs showed that protein binding, GTPase regulator activity, nucleoside-triphosphatase regulator activity, and binding were enriched.

We further studied signaling pathways using the KEGG database. Among the 1000 genes with differentially methylated CpG sites between the gout and control groups, 35 pathways were statistically significant in the KEGG mapping ($p < 0.05$; Supplementary Table S1D). According to *p* value and false discovery rate criteria, the top 10 pathways are listed in Figure 3D. They are mainly related to tumors, such as prostate cancer, acute myeloid leukemia, chronic myeloid leukemia, and non-small-cell lung cancer. They also include metabolism-related signaling pathways, such as phosphatidylinositol signaling system, insulin signaling, and adipocytokine signaling pathways. They also include the chemokine signaling pathway, which may be related to acute attacks of gout. Finally, there is the Notch signaling pathway, which affects multiple processes in normal cell morphogenesis, including differentiation of pluripotent progenitor cells, apoptosis, cell proliferation, and formation of cell boundaries.

TABLE 1 Comparison of clinical laboratory data of two groups

Group	Gout ($n = 50$)	Control ($n = 50$)	*p* value
Age (Y)	43.0 ± 12.6	41.1 ± 11.4	0.235
ALT (U/L)	32.7 ± 23.5	27.3 ± 15.1	0.168
AST (U/L)	21.5 ± 9.9	22.1 ± 9.5	0.734
Cr (μmol/L)	75.6 ± 14.0	72.7 ± 8.7	0.217
UA (μmol/L)	424.8 ± 125.1	343.2 ± 39.8	*<0.001*
Glu (mmol/L)	5.3 ± 0.7	4.6 ± 0.4	*<0.001*
TC (mmol/L)	5.3 ± 1.0	4.8 ± 0.8	*0.009*
HDL (mmol/L)	1.4 ± 0.3	1.4 ± 0.3	0.839
LDL (mmol/L)	3.1 ± 0.9	2.9 ± 0.7	0.268
TG (mmol/L)	2.1 ± 1.5	1.5 ± 0.8	0.050
WBC ($\times 10^9$/L)	7.7 ± 2.2	6.5 ± 1.7	*0.030*

Note: *p* value less than 0.05 was in italic.

Abbreviations: ALT, glutamic-pyruvic transaminase; AST, glutamic oxalacetic transaminase; Cr, creatinine; Glu, blood glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; UA, uric acid; WBC, white blood cell.

3.3 | Hypomethylation of *OPRD1* (Chr1:29,139,121) and high levels of OPRD1 protein in the gout group

Our further analysis showed that the signaling pathways involved in the *OPRD1* gene are the cGMP-PKG signaling pathway, sphingolipid signaling pathway, and neuroactive ligand-receptor interaction. And a search on https://www.genecards.org found that OPRD1 gene was located in plasma membrane and involved in immune response, while it could enable G-protein-coupled receptor activity. We selected the gene *OPRD1* because uric acid may protect the nervous system and may be associated with the pathogenesis of neurological diseases.[17] In addition, *OPRD1* methylation levels are significantly increased in patients with Alzheimer's disease.[18]

We recruited 50 Chinese male gout patients and 50 healthy male controls to investigate the role of *OPRD1* promoter methylation in gout. Among the 11 clinical parameters tested, uric acid, glucose, total cholesterol, and white blood cell counts were higher in the gout group than in the control group ($p < 0.05$; Table 1).

We selected six *OPRD1* sites (pos 1: Chr1:29,139,111, pos 2: Chr1:29,139,117, pos 3: Chr1:29,139,121, pos 4: Chr1:29,139,130, pos 5: Chr1:29,139,143, and pos 6: Chr1:29,139,146) for the detection of methylation levels. The methylation level of *OPRD1* (Chr1:29,139,121) was 12.5 ± 2.5 in the gout group, which was hypomethylation compared with the control group ($p = 0.007$; Table 2, Figure 4A).

The level of OPRD1 protein was significantly higher in the gout samples (237.0 ± 28.6 pg/m) compared with that in the control group (206.9 ± 34.2 pg/ml; $p < 0.001$; Table 2, Figure 4B).

3.4 | *OPRD1* (Chr1:29,139,121) methylation combined with OPRD1 protein level is a promising biomarker for gout

In addition, we analyzed the diagnostic value of *OPRD1* (Chr1:29,139,121) hypomethylation and OPRD1 protein level in peripheral blood by receiver operating characteristic (ROC) analysis. The area under the curve (AUC) of *OPRD1* (Chr1:29,139,121) methylation in gout was 0.664 (0.557, 0.771) with a sensitivity of 64% and a specificity of 68% ($p = 0.005$). The ROC of OPRD1 protein level showed that it was a promising biomarker for gout [AUC = 0.755 (0.660, 0.850), sensitivity of 76%, specificity of 68%, $p < 0.001$]. Furthermore, the AUC of the combination of *OPRD1* (Chr1:29,139,121) hypomethylation and OPRD1 protein level was 0.796 (0.710, 0.883) with a higher sensitivity of 82% and a specificity of 68% ($p < 0.001$; Figure 5).

3.5 | The differential expression of circular RNAs and long non-coding RNAs in gout

We searched for methylation of differentially expressed genes between gout and normal controls from other studies in the Gene Expression Omnibus (GEO) database obtained from https://www.ncbi.nlm.nih.gov/geo. Unfortunately, no relevant information was found. However, we did identify the differential expression of circular RNAs and long non-coding RNAs, suggesting that they may be involved in the pathogenesis of gout through multiple pathways.[19,20]

4 | DISCUSSION

DNA methylation is related to human diseases and aging. Approximately half of human genes have CG-rich sequences, known as CpG islands, in their promoter regions. A variety of factors can affect the activity of promoter regions containing CpG islands.[21] For example, methylated-cytosine binding to transcription factors inhibits its transcription, and CpG island methylation alters the structure of chromatin.[22]

Gene methylation plays a role in the pathogenesis of gout. Ying et al pointed out that *COMT* hypomethylation is associated with the occurrence of gout in men and provided an epigenetic mechanism for the action of *COMT* in gout.[23] Likewise, Li et al showed that hypomethylation of the *CCL2* promoter is a potential biomarker for the diagnosis of gout in Chinese Han males.[24] Yang et al emphasized that *UMOD* methylation might be used to predict the risk of gout.[25]

The high-throughput methylation microarray technique enables rapid analysis of the methylation status of genes by screening

TABLE 2 Comparison of methylation levels in the promoter regions of *OPRD1*, and level of OPRD1 between gout group and control group

Name	Gout ($n = 50$)	Control ($n = 50$)	p value
OPRD1 (Pos 1: Chr1:29,139,111)	4.3 ± 2.0	4.5 ± 2.1	0.645
OPRD1 (Pos 2: Chr1:29,139,117)	5.4 (4.7, 6.2)	5.7 (4.9, 6.4)	0.336
OPRD1 (Pos 3: Chr1:29,139,121)	12.5 ± 2.5	13.9 ± 2.5	**0.007**
OPRD1 (Pos 4: Chr1:29,139,130)	12.4 ± 2.7	13.1 ± 2.6	0.177
OPRD1 (Pos 5: Chr1:29,139,143)	6.2 (5.6, 7.2)	6.0 (5.3, 6.9)	0.240
OPRD1 (Pos 6: Chr1:29,139,146)	11.0 (9.2, 12.4)	10.7 (9.4, 11.8)	0.499
OPRD1 (pg/ml)	237.0 ± 28.6	206.9 ± 34.2	**<0.001**

Bold type indicates statistical significance between the two groups.

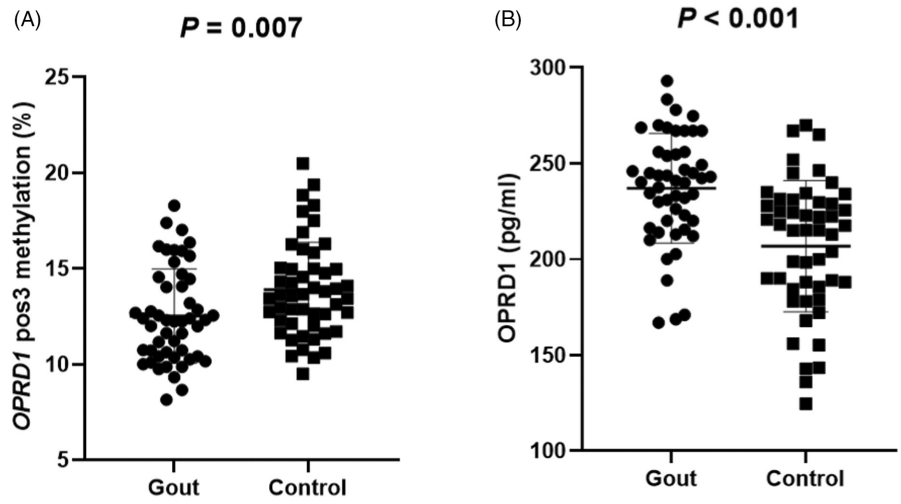

FIGURE 4 Comparison of methylation levels in the promoter regions of OPRD1, and level of OPRD1 between gout group and control group. It showed the methylation level of OPRD1 (Chr1:29,139,121) was 12.5 ± 2.5 in the gout group, and it was hypomethylated in the gout group compared with the control group ($p = 0.007$) (A); while the level of OPRD1 protein was 237.0 ± 28.6 pg/m in the gout samples and 206.9 ± 34.2 pg/ml in the controls, and the level of OPRD1 protein in gout group was higher than in the control group and the difference was statistically significant ($p < 0.001$) (B).

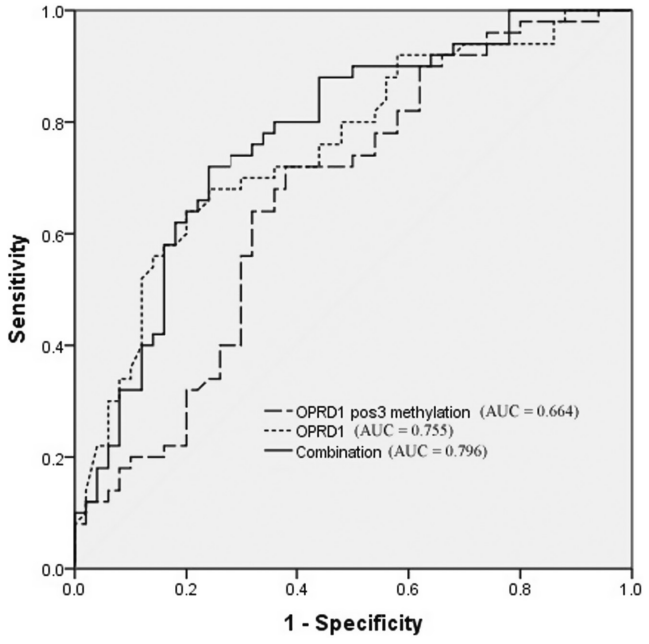

FIGURE 5 The receiver operating characteristic (ROC) curve analysis of gout. It showed the area under the curve (AUC) of OPRD1 (Chr1:29,139,121) methylation in gout was 0.664 (0.557, 0.771) with a sensitivity of 64% and a specificity of 68% ($p = 0.005$). The ROC of OPRD1 protein level showed that it was a promising biomarker for gout (AUC = 0.755 (0.660, 0.850), sensitivity = 76%, specificity = 68%, $p < 0.001$). The AUC of the combination of OPRD1 (Chr1:29,139,121) hypomethylation and OPRD1 protein was 0.796 (0.710, 0.883) with a higher sensitivity of 82% and a specificity of 68% ($p < 0.001$).

small amounts of genomic DNA.[26] We detected 20,426 differentially methylated CpG sites (5719 hypermethylated and 14,707 hypomethylated sites) between the gout and control groups. After GO and KEGG analyses, we selected *OPRD1* for further analysis, which is related to Alzheimer's disease.

OPRD1 encodes the delta-opioid receptor, which participates in the cyclic GMP-protein kinase G signaling pathway, sphingolipid signaling pathway, and neuroactive ligand-receptor interaction, and plays an important role in cognitive function and emotion-driven behavior.[27] Quantitative autoradiography studies have shown that binding of delta-opioid receptors in the amygdala complex and ventral putamen is reduced in the brains of Alzheimer's patients.[28,29] A recent article by Huihui et al pointed out that hypermethylation of *OPRD1* (Chr1: 28,811,734) is associated with an increased risk of Alzheimer's disease.[18] In addition, uric acid is one of the most important antioxidants in the blood and may protect the nervous system from oxidative damage.

In our study, the gout group showed decreased methylation levels at *OPRD1* (Chr1:29,139,121). In addition, OPRD1 protein levels were higher in the gout group than in the control group. Therefore, we suggest that hypomethylation of *OPRD1* (Chr1:29,139,121) might lead to high levels of OPRD1 protein. In addition, we suggest that patients with gout are protected against Alzheimer's disease. In other words, patients with high levels of uric acid may have a reduced risk of developing Alzheimer's disease. Furthermore, there may be a relationship between uric acid and OPRD1 protein.

The ROC analysis of *OPRD1* (Chr1:29,139,121) methylation shows it to be a moderate biomarker for gout diagnosis with moderate AUC, moderate sensitivity, and moderate specificity. OPRD1 protein level, however, is a promising gout diagnosis biomarker with high AUC and high sensitivity. This difference is possibly because the protein level is downstream of gene expression in the mechanism of the disease, and there may be other mechanisms that affect OPRD1 protein level besides gene methylation. The combination of *OPRD1* (Chr1:29,139,121) methylation and OPRD1 protein level

has the highest AUC and sensitivity; therefore, the combination of *OPRD1* (Chr1:29,139,121) methylation and OPRD1 protein level is a potential biomarker for gout diagnosis. Clinically, hypomethylation of *OPRD* and a high level of OPRD protein can indicate the risk of gout earlier than dual-energy CT and joint ultrasound. As a result, interventions can be individualized earlier, providing patients with greater benefits.

In this study, we analyzed the differences in gene methylation and protein levels between patients with gout and control patients. However, there were some limitations to the study. We did not measure mRNA levels, and it would be beneficial to analyze mRNA in future studies. In addition, there is no direct proof that hypomethylation of *OPRD1* (Chr1:29,139,121) leads to a high level of OPRD protein. Finally, the sample sizes were small, and these should be increased in future studies.

In summary, methylation microarray profiling is useful for screening gout-related differentially methylated sites in genomic DNA. Hypomethylation of *OPRD1* (Chr1:29,139,121) might play an important role in gout processes by influencing the level of OPRD1 protein. The combination of *OPRD1* (Chr1:29,139,121) hypomethylation and high OPRD1 protein level is a potential biomarker for gout diagnosis.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts 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.

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