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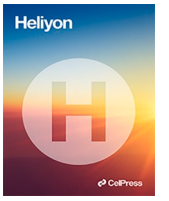

Study on the correlation between suicidal ideation and ABI3BP gene、DPYSL2 gene methylation in pediatric bipolar disorder with depressive episode

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ABSTRACT

Patients with bipolar disorder have a higher risk of suicide than the general population. This study aimed to explore the correlation between suicide and gene methylation, as screened by genome-wide scanning, in children and adolescents with bipolar disorder. A total of 45 children and adolescents with bipolar disorder were divided into a suicidal ideation group (n = 41), a non-suicidal ideation group (n = 4), a low-risk group (n = 12), and a middle-to-high-risk group (n = 33). A pre-experiment was conducted on the suicidal ideation (n = 6) and non-suicidal ideation groups (n = 4). Blood samples were scanned using an Illumina HD 850K microarray, and methylation levels were analysed. Differential methylation sites among the sample groups were screened from the original data, and genes related to suicide were identified. Methylation of the ABI3BP and DPYSL2 genes was detected by pyrophosphate sequencing and statistically analysed. There was a significant difference in age between the low- and middle-risk groups. The results of GO analysis for the suicidal ideation and non-suicidal ideation groups showed that the differential methylation sites were mainly involved in the interferon-γ-mediated signalling pathway, with the main signalling pathways being the inflammatory bowel disease (IBD) pathway and type 1 diabetes mellitus (T1DM) pathway. There were significant differences in the methylation of ABI3BP, HLA-DQB1, HLA-DRB1, AUTS2, SP3, NINJ2, DPYSL2, and other genes between the suicidal and non-suicidal ideation groups. There was also a statistically significant difference in the gene methylation levels between the two groups. However, there was no significant difference in the degree of methylation of the ABI3BP and DPYSL2 genes between the low- and middle-to-high-risk groups. These results suggest that suicidal ideation is correlated with the methylation levels of differentially methylated genes in children with bipolar disorder. However, the severity of suicide risk in paediatric patients with bipolar disorder may not be correlated with the degree of methylation of the ABI3BP and DPYSL2 genes. Therefore, further validation was required.

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1. Introduction

Bipolar disorder is a chronic, disabling, and severe psychiatric disorder characterised by recurrent depressive episodes, manic or hypomanic episodes, somatic and psychiatric comorbidities, and functional impairment [1]. The clinical presentation of paediatric bipolar disorder is similar to that of adult bipolar disorder, with diagnoses based on the same diagnostic criteria. Paediatric bipolar disorder is also characterised by other symptoms, such as disruptive and aggressive behaviour, tantrums, self-injury, and even suicide [2]. These clinical manifestations during the developmental period make it difficult to confirm the diagnosis and provide appropriate treatment [3]. Approximately 2/3 of patients with bipolar disorder have an onset in childhood or adolescence [4], which is a critical period of brain development, plasticity, and maturation [5]. Paediatric bipolar disorder has high co-prevalence [6] and suicide rate [7]. The percentage of global prevalence of paediatric bipolar disorder is 3.9 % [8], with the highest risk of suicide among all adolescent disorders [9]. Holtzman et al. [10] noted that 21.9 % of patients with bipolar disorder developed symptoms at age 12 or earlier, whereas 43 % developed symptoms between the ages of 13 and 18. Weinstein et al. [11] found that the prevalence of suicidal ideation and behaviour in paediatric patients with bipolar disorder ranges from 20 % to 55 %. Up to 50 % of adolescents with bipolar disorder attempt suicide by the age of 18 [12], as suicidal behaviour often peaks during adolescence [9]. Among younger individuals with suicidal ideation, the risk of suicide is greatest in the year following the onset of ideation [13]. Therefore, early identification and intervention for suicidal ideation in this young population before they develop more severe behaviours are critical to prevent morbidity and mortality associated with paediatric bipolar disorder [14]. Kim et al. [15] investigated the frequency of using the paediatric emergency department (PED) among children and adolescents with psychiatric problems. Over a 59-month period, 194 adolescents were admitted, 91 of whom were admitted for attempted suicide or non-suicidal self-injury.

Clinical and molecular findings reveal that suicidal behaviour in mood and affective disorders has familial and environmental bases [16]. A large body of medical evidence suggests that suicide is associated with epigenetics, which refers to genomic modifications that do not alter DNA sequences and can be influenced by environmental stimulation [17]. Epigenetics is an adaptive mechanism that can modulate the stress response through subtle modifications in gene expression [18]. In particular, the addition of a methyl group to DNA, mainly on cytosine-guanine dinucleotide (CpG), has been proposed as a mechanism by which early-life experiences may become "embedded" in the genome [19]. Numerous studies showed that DNA methylation plays an important role in neural regeneration and remodelling [20]. Jokinen et al. [21] divided participants into high-risk and low-risk groups according to the severity of suicidal behaviour and found that altered methylation of the CRH gene was associated with the severity of suicide risk in adults. Li et al. [22] used the Global Assessment Scale for Suicide Risk (NGASR) to assess suicide risk in participants and divided 107 patients with MDD into two groups: high risk (NGASR$\geq$9) and low risk (NGASR$<$9). They found that reduced serum VGF levels in MDD patients were associated with the severity of suicide risk.

In recent years, several studies showed that methylation alterations of genes associated with suicidal ideation in patients with psychiatric disorders include the CACNA1C gene, HLA complex 9 gene (HCG9), membrane palmitoylated protein (MPP4), synapsins (SYN1, SYN2, and SYN3), and CRH. However, most studies have focused on adults, and studies related to suicide risk and gene methylation in paediatric patients with bipolar disorder are rare. There are two important limitations to most studies so far: (1) the cross-sectional design limits inferences about the aetiological processes; (2) the use of adult samples introduces confounding factors [5]. Therefore, in this study, we used Illumina HD 850K microarray technology to screen genome-wide differentially methylated genes in 10 paediatric bipolar disorder patients with and without suicidal ideation. After screening genes associated with suicide in paediatric patients with bipolar disorder (ABI3BP and DPYSL2), we hypothesized that the severity of suicide risk in paediatric patients with bipolar disorder was correlated with the degree of methylation of these screened genes. We used pyrophosphate sequencing to sequence the ABI3BP and DPYSL2 genes of 45 participants with paediatric bipolar disorder to verify the correlation between the severity of suicide risk and the methylation of the screened genes (ABI3BP and DPYSL2). We hope that this study will provide a reference for studying the neuropathophysiological mechanisms and theoretical basis for predicting and intervening in suicidal ideation in paediatric patients with bipolar disorder.

2. Methods

2.1. Participants

All participants were patients with depressive episodes of bipolar disorder who were first seen in the Department of Clinical Psychology at the People's Hospital of Xinjiang Uygur Autonomous Region between December 2018 and May 2020, regardless of gender. A total of 45 patients were enrolled in this study.

The criteria for enrolment in this study were as follows. (1) All participants were required to meet the diagnostic criteria for bipolar disorder (F31) in the International Statistical Classification of Diseases and Related Health Problems, 10th edition (ICD-10). F31 bipolar affective disorder is characterised by two or more episodes in which the patient's mood and activity levels are significantly disturbed, with this disturbance consisting on some occasions of an elevation of mood and increased energy and activity (hypomania or mania) and on others of a lowering of mood and decreased energy and activity (depression). Repeated episodes of hypomania or mania only are classified as bipolar. (2) The total score of the Hamilton Depression Inventory (HAMD) [23] was $>$20. (3) Each participant was clinically diagnosed by two or more experienced attending psychiatrists. If consensus on a diagnosis could not be reached, a third clinician in the role of a psychiatric chief physician or associate chief physician would establish the diagnosis. (4) All participants volunteered to participate in the study and signed an informed consent. (5) The age ranged from 12 to 18 years.

The exclusion criteria were as follows: (1) history of head trauma, organic brain disease, epilepsy, or other known neurological

disorders that can cause mental disorders, such as Parkinson's disease, dementia, or multiple sclerosis; (2) severe physical illness (especially endocrine disorders related to mood changes and immune system disorders, such as rheumatic diseases); (3) history of psychoactive substance abuse or dependence; (4) IQ $\leq$ 80; (5) no treatment in the month before admission, including any medical treatment for psychiatric conditions, psychotherapy, or physicotherapeutics such as repetitive transcranial magnetic stimulation therapy.

2.2. Measures

The Suicidal Ideation of Self-Evaluation Scale (SIOSS) is a self-reported measure suitable for all ages in primary school education. This scale assesses despair, optimism, sleep, and concealment. The total suicidal ideation score was calculated by adding the scores for these factors. The higher the total score, the stronger the suicidal ideation. If the total score was <12, the patient was considered as having no suicidal ideation. A concealment factor score of ≥4 was considered an unreliable measurement and was excluded from data analysis. The SIOSS had good reliability and validity among the Chinese population [24,25].

The Nurses' Global Assessment of Suicide Risk (NGASR) scale is a commonly used psychiatric scale to assess suicide risk. It is widely used in hospitals and is prospectively designed for patients with psychiatric disorders hospitalised in a psychiatric ward. The NGASR was used at admission and discharge in all patients under 18 years [26]. The scale categorises suicidal risk as low with a score of less than or equal to 5, moderate with a score of 6–8, high with a score of 9–11, and super high with a score of 12 [27].

2.3. Grouping of participants

All 45 participants were divided into two groups according to their Self-Assessment of Suicidal Ideation Scale (SIOSS) scores. A score greater than or equal to 12 indicated suicidal ideation, whereas a score less than 12 indicated no suicidal ideation [24]. As a result, 41 participants were placed in the suicidal ideation group (SIOSS score ≥12) and 4 participants in the non-suicidal ideation group (SIOSS score <12). Six participants from the suicidal ideation group (n = 6) and four from the non-suicidal ideation group (n = 4) were selected for the pretest.

Initially, we planned to use a single scale for grouping. Therefore, when we collected 10 samples, we grouped them according to suicidal ideation to conduct a pre-experiment. Ultimately, 45 samples were collected, and most participants reported suicidal ideation. Due to the large difference in sample size between the two groups, we decided to further group them according to suicide risk using the NGASR scale.

All 45 participants were grouped according to their NGASR scores. Finally, 12 participants were placed in the low-risk group (NGASR <6) and 33 participants in the medium-to-high-risk group (NGASR ≥6).

This study was approved by the Ethics Committee of the People's Hospital of the Xinjiang Uygur Autonomous Region. All participants voluntarily participated in the study, and written informed consent was obtained from both the participants' legal guardians and the participants themselves.

2.4. Detection method and data processing

4 ml of elbow venous blood was collected from participants, placed in a 5 ml ethylenediaminetetraacetic acid (EDTA)-Na2 anticoagulation tube, labelled, and stored in a refrigerator at −80 °C. DNA samples were extracted to ensure its purity: OD260/280 values were between 1.7 and 2.0, RNA was removed, concentration of DNA samples was ≥55 ng/μl, and the total amount of samples was >5 μg. The samples were then dissolved in TE and transported at low temperature (−20 °C), with the mouths of the tubes sealed with parafilm to prevent contamination during transport.

DNA samples were extracted using the TIANGEN Blood Genomic DNA Extraction Kit and treated with thionite using the EZ DNA Methylation Gold Kit (Zymo, USA). The process included denaturation, base denaturation, whole genome amplification, hybridisation, washing, extension, and staining. Methylation was detected using the Infinium Human Methylation 850 BeadChip (Illumina, USA). The microarray contained 853,307 methylation sites across the human genome. The methylation chip is a probe designed to target one site, and a gene may detect multiple sites. However, the sites selected were those with the most significant differences in pyrosequencing. Pyrosequencing is approximately 60bp in length, and if this region contains multiple methylated sites, it can be detected simultaneously. However, these few genes have only one methylated site measured at this time.

Microarray pairs were scanned using an iSan scanner. PCR amplification and pyrophosphate sequencing of the selected SNP loci were performed. These sites were located mainly in the promoter and 500 BP region upstream of the transcription start site. Methylated primers were designed using Methyl Primer Express v1.0.

The HLA-DQB1 and HLA-DRB1 genes were not amplified because of the contiguous poly a structure in the genetically examined segment. In addition, attempts to swap several primer pairs failed to amplify the product. One sample had blood clots in whole blood, and the DNA quality was poor. Therefore, the product concentration was low during subsequent PCR amplification. There were heteropeaks in the sequencing, and there were no results from inaccurate sequencing results.

2.5. Statistical analysis

Data for the samples were compared using SPSS26.0 for statistical analysis. The measurement data were expressed as mean ± standard deviation ($\overline{x} \pm s$). A *t*-test was used for comparison between the two groups, conforming to normal distribution. Count data

were expressed as rates (%). X^2 test or Fisher's exact probability method was used to compare count data between the two groups, conforming to normal distribution.

Whole-blood DNA methylation sites were extracted from the raw data using GenomeStudio software to obtain signal values. The raw data were then imported into Genomestudio software for standardisation using background normalisation and average normalisation. The results were screened for differentially methylated genes, and the two groups used for comparison were set up with three or more replicates using the *t*-test, according to Illumina's recommended screening criteria for this microarray. Methylation analysis was performed to extract methylation information for individual loci in individual samples and sample groups, and it provided intensity, beta, and detection data. The samples were not compared with each other, as they were differentially methylated. The beta value was used to estimate the methylation level of the CpG locus using the ratio of the intensities of the methylated and unmethylated alleles. Genes with methylation differences >17 % ($|\triangle\beta| > 0.17$) between samples and $P < 0.05$ were classified as differential candidates. β values were calculated as follows:[1.] [28].

Differential Methylation Analysis Algorithms.

All differential methylation analysis algorithms were used to compare a group of samples (referred to as the condition group) with a reference group. This comparison was made using the Illumina Custom model, Mann–Whitney model, and *t*-test model. The Illumina Custom model operates with the assumption that the methylation value β is normally distributed among replicates, corresponding to a set of biological conditions. The variation in the estimate of β is a function of β. The function was estimated for all values of β by repeatedly measuring loci with known methylation fractions ranging from 0 to 1 and then fitting a parabola to the standard deviation as a function of β. The standard deviation estimate was then given by $^{2.}s = A\beta 2 + B\beta + C$, where for GoldenGate and Infinium Methylation, A = −0.1511, B = 0.1444, and C = 0.01646 and for VeraCode Methylation, A = −0.1582, B = 0.1554, and C = 0.00756. We produced *p* values using the following approach: *z* is the two-sided tail probability of a standard normal distribution. The differential score for a probe was computed as follows. For a locus with multiple probes, the differential scores for probes were averaged, and the concordance value between the probes was reported. The Mann–Whitney model produced an exact *p* value if a normal approximation with continuity correction was used. The differential scores were computed as previously described for the Illumina Custom model. For the *t*-test model, when either the reference group or condition group contained at least two samples, the variance was estimated across replicate samples. Otherwise, the variance was estimated from bead-to-bead variation. We used t-tests with the assumption of equal variance. The differential scores were computed in the same manner as the Illumina Custom model.

Gene methylation levels in pre-experimental groups and methylation rates in all samples were compared using SPSS19.0 statistical software to store and analyze the data. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Two independent samples with normally distributed means were analysed using an independent sample *t*-test or ANOVA. Data not conforming to normal distribution were analysed using the rank sum test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Sample characteristics

There were no statistically significant differences between the two groups in terms of gender, education level, or socioeconomic status ($P > 0.05$). However, we found a significant difference in age between the low- and medium-to-high-risk groups (Tables 1 and 2).

3.2. Quality control results of methylation 850K BeadChip detection

Whole-blood genomic DNA samples from 6 patients with suicidal ideation and 4 without were analysed for methylation levels using Illumina's human 850k methylation microarray, which interrogates >853,000 CpG sites with a comprehensive coverage of CpG islands, promoters, coding regions, open chromatin, and enhancers. In addition, CpG sites outside CpG islands, known as DNA methylation sites, deoxyribonuclease hypersensitive sites, and miRNA promoter regions were included. Infinium I and II probe designs were used to maximise the detection range. The 850k chip conveys the design concept of the 450k chip to the functional elements of

Table 1
Comparison of the general date of the subjects.

General Situation	the suicidal ideation group (n = 6)	The non- suicidal ideation group (n = 4)	*t*/Fisher's	*P*
Age	15.83 ± 1.47	15.75 ± 1.50	0.087	0.933
Gender			–	0.286
Female	5 (83.3 %)	2 (50.0 %)		
Male	1 (16.7 %)	2 (50.0 %)		
Education Level			–	0.592
Middle School	4 (66.7 %)	2 (50.0 %)		
High School	2 (33.3 %)	2 (50.0 %)		
The economic situation			–	1.000
Very poor	1 (16.7 %)	0		
Moderate	2 (33.3 %)	3 (75.0 %)		
Better	3 (50.0 %)	1 (25.0 %)		

Table 2
Comparison of the general date of the subjects between low-risk group and medium-high-risk group of suicide.

General Situation	low-risk group (N = 12)	medium-high-risk group(N = 33)	t/X^2/Fisher's	*P*
Age	14.83 ± 1.70	16.12 ± 1.54	2.420	0.020
Gender			–	1.000
Female	9(75.0 %)	25(75.8 %)		
Male	3(25.0 %)	8(24.2 %)		
Education Level			2.493	0.398
Primary school	1(8.3 %)	0		
Middle School	6(50.0 %)	17(51.5 %)		
High School	5(41.7 %)	16(48.5 %)		
The Economic Situation			3.332	0.173
Bad	0	6		
Moderate	8(66.7 %)	22(66.7 %)		
Good	4(33.3 %)	5(15.2 %)		
Family history of mental illness			–	0.682
With	3(25.0 %)	6(18.2 %)		
Without	9(75.0 %)	27(81.8 %)		
Only child			0.895	0.501
Yes	7(58.3 %)	14(42.4 %)		
No	5(41.7 %)	19(57.6 %)		

the genome, with unprecedented comprehensive coverage of CpG islands and genes. The table statistics demonstrate the coverage and distribution of the 850k chip to different chromosomes and functional elements of the genome. DNA extraction, conversion, amplification, and sulphite handling were performed strictly according to the Illumina Infinium Human Methylation 450 BeadChip Operator Manual, and each QC report assay met Illumina's quality requirements. The hybridisation probe QC plots showed that the log2 (background value) generated by the green probe in the negative reference was close to 0, whereas the sample log2 (signal value) generated by the red probe was >12. Probe labelling was successful, with a difference in log2 (background value) and log2 (signal value) of over two orders of magnitude. The elution experiments were also successful (Fig. 1A and B).

3.3. Distribution characteristics of differentially methylated sites along chromosomes

Of the 866,151 loci detected by the Illumina HD 850k chip, based on a differential score of less than −13 or greater than 13 and Delta_beta of more than 0.17 or less than −0.17, $P < 0.05$, a total of 267 differentially methylated loci were filtered out, including 152 hypermethylated and 115 hypomethylated loci. These 92 differentially methylated loci were distributed along chromosomes 1 to 22 and X. The most differentially methylated regions were distributed on chromosome 1, as detailed in Fig. 2.

3.4. Comparison of methylation levels between groups with and without suicidal ideation

Using the Infinium Human Methylation 850 BeadChip to scan the blood samples of 10 paediatric patients with bipolar disorder, we found 267 differentially methylated loci in the group with suicidal ideation compared with the group without suicidal ideation, indicating a difference in gene methylation levels between the two groups. Table 3 shows that differences ($F = 35.046$, $P = 0.000 < 0.05$) in the gene methylation levels between the group with (n = 6) and the group without suicidal ideation (n = 4) are statistically significant.

All genes on the 267 differentially methylated loci screened were entered into the PubMed database separately, and their correlation with suicide was determined. Seven genes were identified: ABI3BP, HLA-DQB1, HLA-DRB1, AUTS2, SP3, NINJ2, and DPYSL2. After unsuccessful PCR amplification of the HLA-DQB1 and HLA-DRB1 genes, we did an extensive literature review and research and decided to perform pyrosequencing of the ABI3BP and DPYSL2 genes.

3.5. Results of functional enrichment analysis

The results of GO analysis suggest that differentially methylated sites are mainly involved in interferon-γ-mediated signaling pathways and other functions (Fig. 3,Table 4). Pathway analysis shows that the signaling pathways are mainly inflamma -tory bowel disease (IBD) pathways and type I diabetes pathways (Fig. 4,Table 5).

3.6. Screening of differentially methylated regions between sample groups by gene annotation classification and CpG island annotation classification

The gene annotation classification contained a total of six regions: TSS200, TSS1500, 1stExon, 5′UTR, 3′UTR, and genebody. The CpG island annotation classification contained five regions: N shore, N shelf, CpG island, S shelf, and S shore. The research results showed that, according to gene annotation classification, there were a total of 14 methylated regions in the TSS1500 region, 8 in the TSS200 region, 15 in the 5′UTR region, 3 in the 1stExon region, 113 in the genebody region, and 8 in the 3′UTR region in the group with suicidal ideation compared to the group without. According to the CpG island annotation classification, there were 14, 28, 17, 18, and

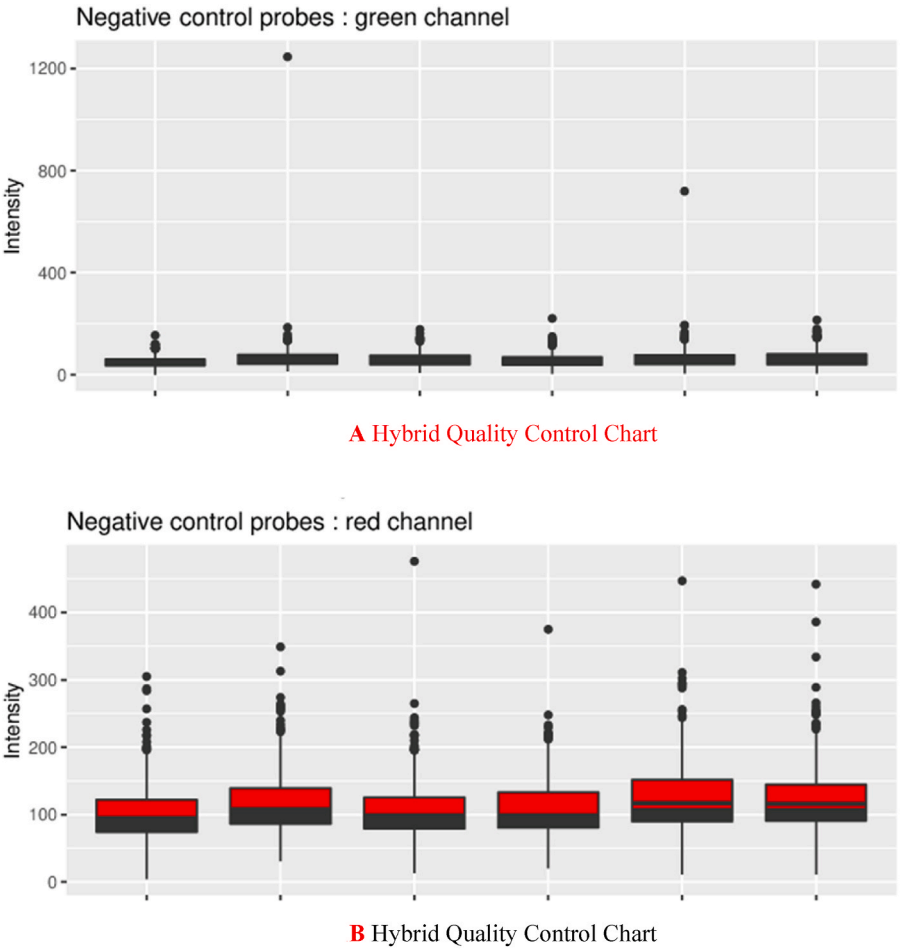

Fig. 1. A Hybrid Quality Control Chart, B Hybrid Quality Control Chart. Note: the horizontal axis in the figure is log2 (signal value) and the vertical axis is the sample name. Red fluorescence indicates log2 (signal value) of unmethylated sites and green fluorescence indicates log2 (signal value) of methylated sites.

15 differentially methylated regions in N shelf, N shore, CpG island, S shore, and S shelf, respectively, in the suicidal ideation group compared to the non-suicidal ideation group. The results of screening the differentially methylated regions among sample groups (P< 0.05, | Δβ | >0.17) are detailed in Table 6.

3.7. Results of cluster analysis

In this study, 267 differentially methylated sites were subjected to cluster analysis. As shown in Fig. 5, the experimental results for this gene chip are ideal. Each row in the heat map represents a bit, each column represents a sample, and the top tree structure represents clustering between different samples. The heat map shows that the group with suicidal ideation is clustered together and the group without suicidal ideation is also clustered together, indicating that the samples within each group are closely related and well clustered. The tree structure on the left represents the clustering relationship between the different sites. There are more methylated sites in the group with suicidal ideation and fewer methylation sites in the group without suicidal ideation (Fig. 5).

3.8. PCR amplification results of the ABI3BP and DPYSL2 genes

A random sample of 5ul PCR product was subjected to 1 % agarose electrophoresis. Electrophoresis was observed at 150V, 100 mA for 20 min. The PCR product bands were single and bright with no spurious bands, indicating good primer specificity, and the PCR product size was in accordance with the expected size (Fig. 6A and B; Fig. 7A, B, and 7C). The product size of the DPYSL2 gene was

Fig. 2. Analysis of differential methylation sites on chr.

Table 3
Analysis of variance of gene methylation level between suicidal ideation group and non-suicidal ideation group.

Grouping	Scale Scores	Gene Methylation Level
Non-suicidal Ideation Group (n = 4)	9.75 ± 0.957	7.00 ± 1.826
Suicidal Ideation Group (n = 6)	20.167 ± 1.472	19.33 ± 3.829
F	153.374	35.046
p	0.000	0.000

Note: F-value is the statistic of ANOVA.

143bp, while that of ABI3BP was 139bp (Table 7).

3.9. Statistical results of the methylation rate

The genes of the 45 samples were sequenced and analysed separately, and sequencing peak plots were obtained (Figs. 8 and 9), in which one sample in the medium-to-high-risk group was sequenced unsuccessfully. We performed covariance analysis to control for the potential confounding effect of age on the outcome variable and analysed the correlation between the methylation rates of the ABI3BP and DPYSL2 genes and suicide risk. The methylation rates of the ABI3BP and DPYSL2 genes in the low-risk (n = 12) and medium-to-high-risk groups (n = 32) are detailed in Table 8. The differences between the two groups were not statistically significant ($F_{ABI3BP} = 00.598$, $F_{DPYSL2} = 1.195$, $P > 0.05$) .

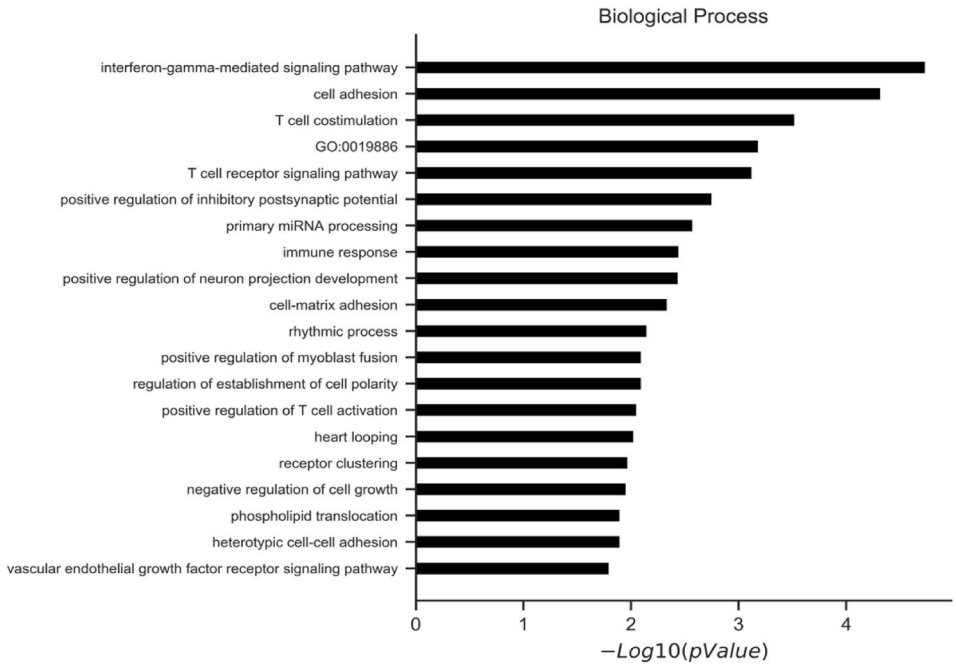

Fig. 3. GO analysis between suicide group and non-suicide group.

Table 4
GO analysis between suicide group and non-suicide group.

Term_ID	Term_description	*P*-Value	-log10 (pvalue)	GeneSymbols
GO:0060333	interferon-gamma-mediated signaling pathway	0.0000182773830930683	4.738085985	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1; MID1
GO:0007155	cell adhesion	0.0000477528561376382	4.321000648	ROBO1; SPON1; AATF; ITGB2; PKP1; CLCA2; MADCAM1; COL6A3; FAT1; SIGLEC5; CDH4; SIGLEC14
GO:0031295	T cell costimulation	0.000299744	3.52325005	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	0.000650361	3.186845464	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1
GO:0050852	T cell receptor signaling pathway	0.000748284	3.125933628	HLA-DRB5; HLA-DRB1; PSMB9; HLA-DQA1; HLA-DPA1; HLA-DQB1
GO:0097151	positive regulation of inhibitory postsynaptic potential	0.001759865	2.754520645	NTSR1; RIMS2
GO:0031053	primary miRNA processing	0.002663682	2.574517593	MADCAM1; ADAMTS12; ITGB2; NID1
…	…	…	…	…

4. Discussion

Since the exact aetiology and pathogenesis of bipolar disorder cannot be elucidated and symptoms are atypical in early episodes, the disorder has a high rate of misdiagnosis [29]. Moreover, patients with bipolar disorder are at a higher risk of suicidal behaviour than patients with other psychiatric disorders [30] or the general population [31], and it usually begins in adolescence or adulthood. In our study, we found that the older the child, the higher the suicide risk ($p < 0.05$). Suicide is the leading cause of death among people aged 15–24 years worldwide [32]. By screening children younger than 10 years of age, Mary et al. [33] found that older patients presented with suicide attempts or ideation more frequently than younger patients. Birmaher et al. [34] compared 173 children with bipolar disorder (aged <12 years), 101 adolescents with childhood-onset bipolar disorder, and 90 adolescents with adolescent-onset bipolar disorder and found that in both groups (compared to children), symptoms such as jumpy thinking, increased productivity, and decreased judgment were observed during manic episodes. During depressive episodes, symptoms were more severe and suicide attempts occurred more frequently. In this study, a genome-wide scan of 10 paediatric bipolar disorder patients with and without suicidal ideation using Illumina HD 850K microarray technology showed 267 differentially methylated loci in the group with suicidal ideation compared to the group without, indicating a difference in gene methylation levels between the two groups ($p < 0.05$). De Luca et al. [35] found that, in patients with severe psychiatric disorders who committed suicide, gene methylation levels were slightly lower

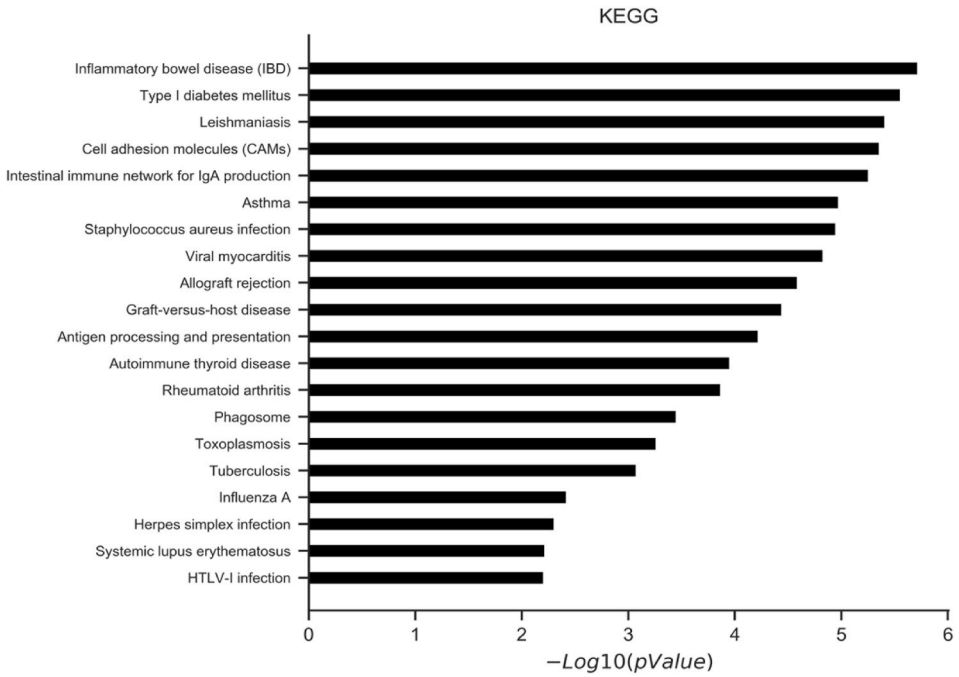

Fig. 4. Pathway analysis between suicide group and non-suicide group.

than in those who did not commit suicide. However, in patients with bipolar disorder, there was no significant difference between those who attempted suicide and those who did not.

The results of differentially methylated genes in children with bipolar disorder in the group with suicidal ideation and the group without were analysed using the GO database, suggesting that differentially methylated loci were mainly involved in functions such as interferon-γ-mediated signalling pathways. Pathway analysis of the differentially methylated loci using the KEGG database showed that they were mainly involved in inflammatory bowel disease (IBD) and type I diabetes pathways. Gaine et al. [36] found that calcium signalling channels are the most important channels in patients with bipolar disorder who commit suicide.

Inflammatory bowel disease (IBD) is a chronic disease of the GI tract [37]. Some studies showed that immune-mediated inflammatory diseases are often comorbid with depression, anxiety disorders, and bipolar disorder, which are common mental disorders (CMD) [38]. Several studies suggested that genetic factors, neurobiological alterations, social factors, and inflammation play an important role in the pathogenesis of BD [39,40]. A Danish population-based study showed that Crohn's disease is associated with an increased risk of BD [41]. A cross-sectional study showed that IBD and BD may have similar biological pathogenesis. Patients with IBD are 2.1 times more likely to develop bipolar disorder than healthy individuals [42]. Several studies suggested that inflammatory cytokines play a key role in the pathogenesis of IBD [43]. Other studies highlighted the potential involvement of immune inflammatory processes in the aetiology and development of bipolar disorder and demonstrated an increased risk of suicide in patients with CMD associated with comorbid immune-mediated inflammatory diseases [44,45]. The above findings, combined with the results of this experimental study, led us to infer that bipolar disorder and IBD may share the same pathogenic factors, such as immune inflammation-related pathways.

In recent years, studies have found that populations with insomnia exhibit significant metabolic changes, reflecting increased insulin secretion, increased risk of diabetes, and abnormal calcium signalling pathways. One of the most important single nucleotide polymorphisms (SNPs) in insomnia-related genes is associated with bipolar disorder [46]. Calkin et al. [47] suggested that bipolar disorder and type II diabetes mellitus (T2DM) may share the same pathophysiological origin and may have common genetic links and epigenetic processes. Compared to the general population, patients with bipolar disorder have a threefold increased risk of developing T2DM [48]. The sample in this experiment consisted of children and adolescents because T2DM is commonly found in adults, while T1DM is commonly found in minors. As this experimental study found that differentially methylated loci in paediatric bipolar disorder are mainly involved in the type I diabetes pathway, it is not difficult to speculate, in combination with the above findings, that paediatric bipolar disorder and T1DM may share the same epigenetic process. Cluster analysis revealed significant methylation differences between the groups with and without suicidal ideation. More hypermethylated loci were found in the group with suicidal ideation than in the group without. However, Gaine et al. [36] found more hypomethylated than hypermethylated differentially methylated regions in patients with bipolar disorder.

The GO analysis results suggest that almost all of the top five signalling pathways involved in differentially methylated loci are immune-related signalling pathways, all of which involve the HLA-DQB1 and HLA-DRB1 genes. The pathway analysis results showed that almost all of them were immune-related disease signalling pathways, and most of them involved the HLA-DQB1 and HLA-DRB1

Table 5
Pathway analysis between suicide group and non-suicide group.

Term_ID	Term_description	*P*-Value	-log10(pvalue)	GeneSymbols
path:hsa05321	Inflammatory bowel disease (IBD)	1.905×10^{-6}	5.720100406	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1; SMAD3; IL4R
path:hsa04940	Type I diabetes mellitus	2.776×10^{-6}	5.556613388	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1; PTPRN2
path:hsa05140	Leishmaniasis	3.900×10^{-6}	5.408893764	HLA-DRB5; ITGB2; HLA-DRB1; MAPK14; HLA-DQA1; HLA-DPA1; HLA-DQB1
path:hsa04514	Cell adhesion molecules (CAMs)	4.381×10^{-6}	5.358401457	HLA-DRB5; ITGB2; HLA-DRB1; NEGR1; HLA-DQA1; MADCAM1; HLA-DPA1; HLA-DQB1; CDH4
path:hsa04672	Intestinal immune network for IgA production	5.536×10^{-6}	5.256831582	HLA-DRB5; HLA-DRB1; HLA-DQA1; MADCAM1; HLA-DPA1; HLA-DQB1
path:hsa05310	Asthma	10.582×10^{-6}	4.975412973	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1
path:hsa05150	*Staphylococcus aureus* infection	11.220×10^{-6}	4.950018454	HLA-DRB5; ITGB2; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1
path:hsa05416	Viral myocarditis	14.786×10^{-6}	4.830154913	HLA-DRB5; ITGB2; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1
path:hsa05330	Allograft rejection	25.840×10^{-6}	4.587716779	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1
path:hsa05332	Graft-versus-host disease	36.098×10^{-6}	4.442520437	HLA-DRB5; HLA-DRB1; HLA-DQA1; HLA-DPA1; HLA-DQB1

Table 6
Analysis of differential methylation region according to the Gene/Cpg island between suicide ideation group and non-suicide ideation group.

Region	Hypomethylation	Hypermethylation	Total
1stExon	2	1	3
3′UTR	2	6	8
5′UTR	5	10	15
Body	55	58	113
TSS1500	0	14	14
TSS200	0	8	8
Island	9	8	17
N_Shelf	5	9	14
N_Shore	12	16	28
S_Shelf	8	7	15
S_Shore	4	14	18

Note: Region: 1.gene/CpG island annotation region name, Total: total number of differentially methylated regions in the suicide group compared with the non-suicide group; Hypomethylated: total number of differentially hypomethylated regions in the suicide group compared with the non-suicide group; Hypermethylated: total number of differentially hypermethylated regions in the suicide group compared with the non-suicide group. 2.Due to the presence of methylation in different regions of the methylation chip, such as TSS, 1stExon, 3′UTR, 5′UTR, and GeneBody methylation, enrichment analysis was conducted on the methylation status of different regions with different results in the chip to analyze the GO functions that may be affected by methylation in different regions.

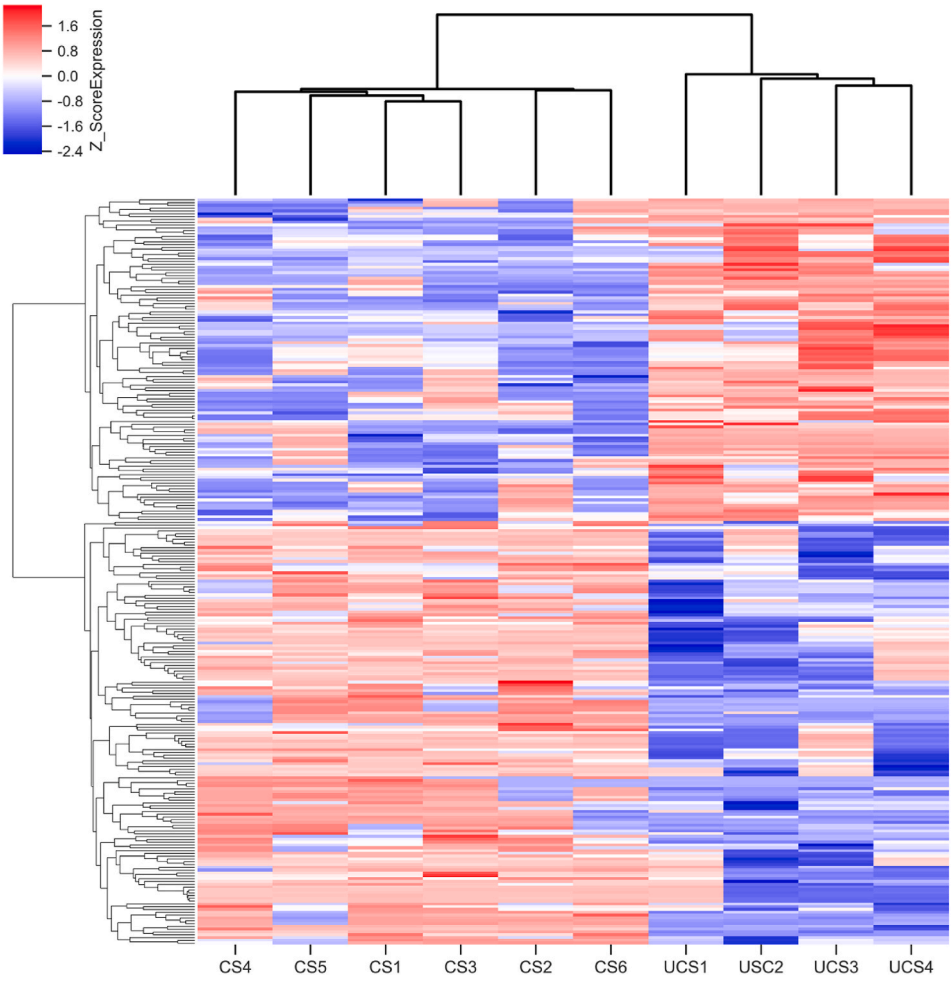

Fig. 5. Heatmap of differential methylation sites between suicide group and non-suicide group. Note:Red represents high methylation, and blue represents the low methylation.

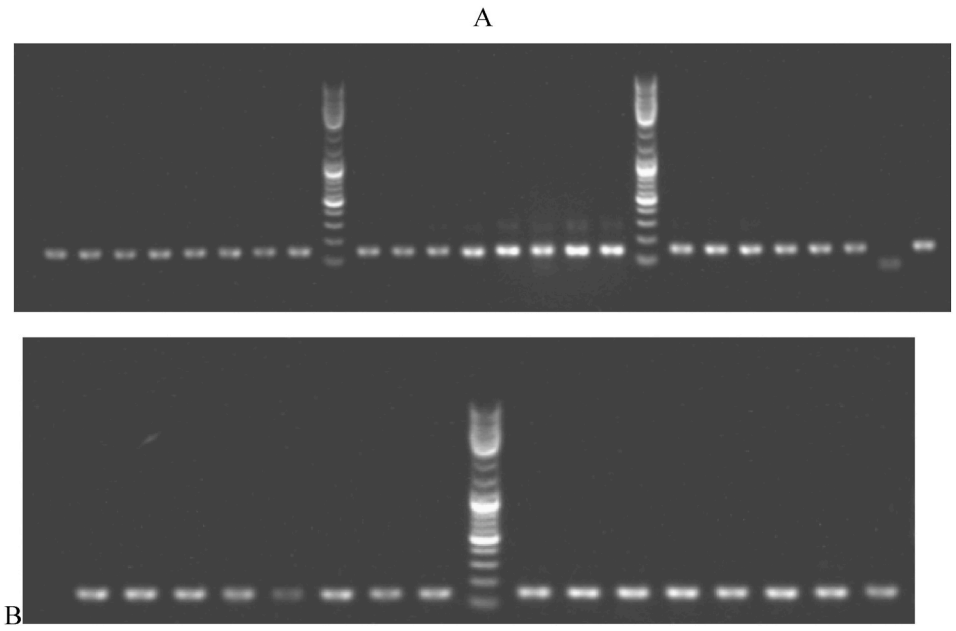

Fig. 6. The PCR product size of the DPYSL2 gene was 143bp (A、B) .

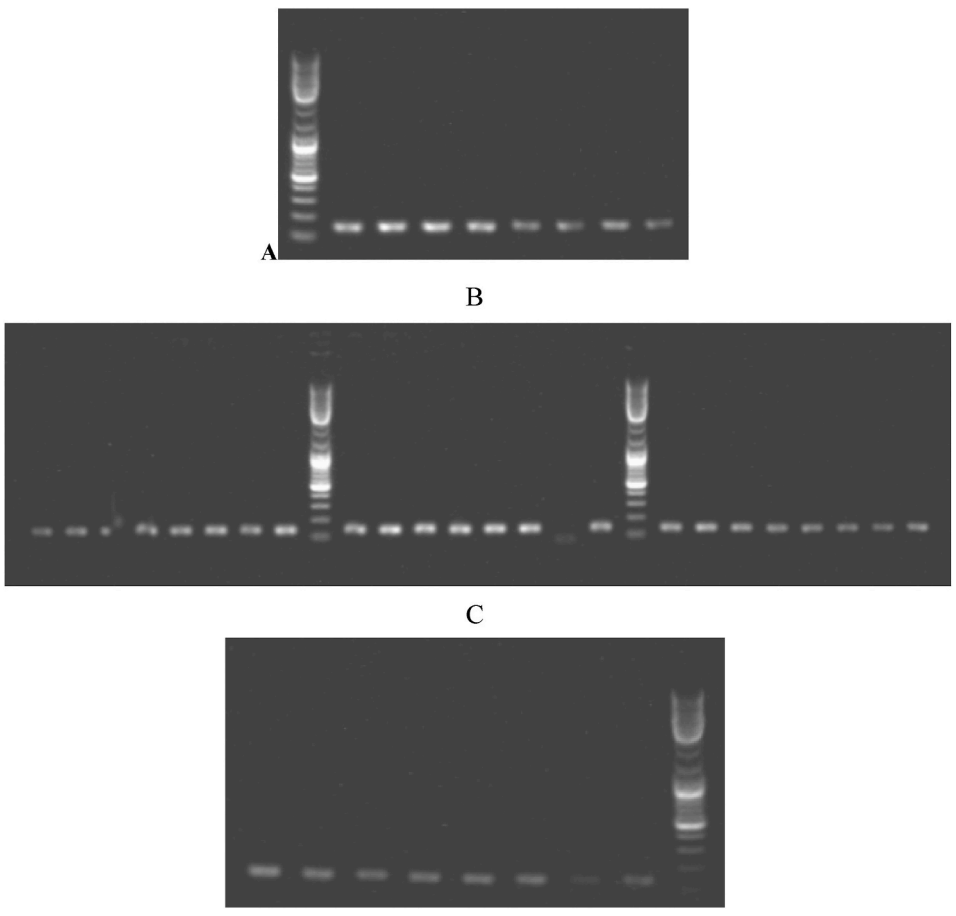

Fig. 7. The PCR product size of the ABI3BP gene was 139bp (A、B、C) .

Table 7
Primer sequence.

	Primer name	Primer sequence	Product size
Upstream primers	PM484-ABI3BP-2F	GTAAGTAGTTTGATGTTTTATGTTTTAAA	151bp
Downstream primers	PM484-ABI3BP-2BR	TTCACACATTTAAACTTTAAATCTCTAC 5-terminal biotin labeling	
Sequencing primers	PM484-ABI3BP-2FS	TTTAAATATGATTTGTTTTATTATA	
Upstream primers	PM484-DPYSL2-1F	ATATATGAGGGTTTTGGATTAGATG 5-terminal biotin labeling	143bp
Downstream primers	PM484-DPYSL2-1BR	AATCTAAATATCCACAATTAAACCAC	
Sequencing primers	PM484-DPYSL2-1FS	AATTAAACCACTTAAAATCTC	

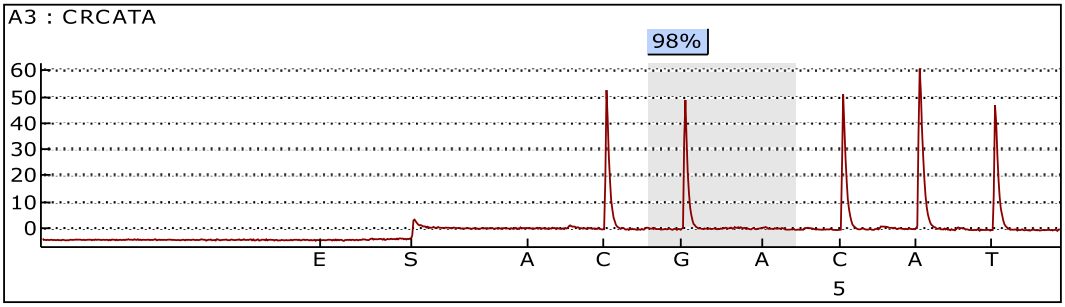

Fig. 8. Sequencing peak map of CpG1 site of DPYSL2 gene (one sample) .

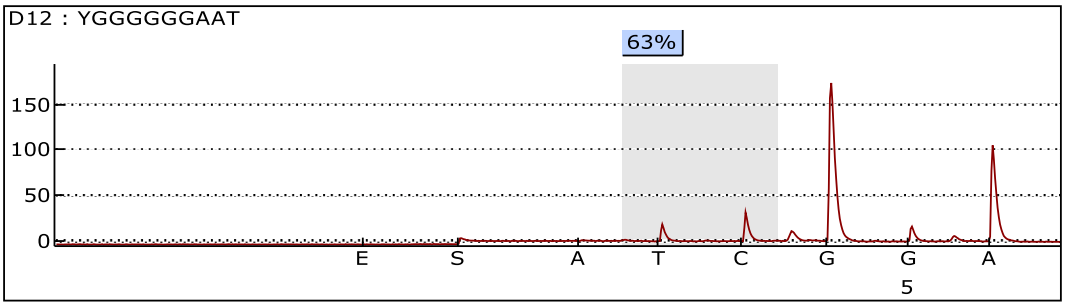

Fig. 9. Sequencing peak map of CpG1 site of ABI3BP gene (one sample) .

Table 8
Analysis of CpG island methylation rate of ABI3BP gene and DPYSL2 gene in low risk group and medium-high risk group of suicide.

Gene	CpG Island	Grouping	CpG island methyla -tion rate (%)	*F*	*P*
ABI3BP	CpG1	Low-Risk Group (n = 12)	29.833 ± 15.147	0.598	0.444
		Medium-High-Risk Group of Suicide (n = 32)	25.906 ± 19.466		
DPYSL2	CpG1	Low-Risk Group (n = 12)	74.667 ± 25.649	1.195	0.281
		Medium-High-Risk Group of Suicide (n = 32)	82.250 ± 23.949		

Note: *F* value is the statistic of Covariance analysis.

genes. The major histocompatibility complex (MHC) region located on the short arm of chromosome 6 (6p21.3–22.1) is the most polymorphic and densely populated region of the human genome. HLA molecules regulate specific adaptive immune responses through antigen processing and presentation, intercellular recognition, and self- and non-self-discrimination. The MHC class II molecules HLA-DRB1, HLA -DQB1, and HLA-DPB1 play a key role in initiating humoral immune responses. In genome-wide association studies (GWAS), the MHC is the locus with the highest statistical significance. Tamouza et al. [49] found that the HLA-DRB03 and HLA-DQB102 subtypes were significantly more common in patients with bipolar disorder and suicidal behaviour. Matei et al. [50] studied the HLA genotyping of individuals with a history of suicide attempts and those who never exhibited such behaviour and found that the genotype with the highest risk of suicidal behaviour was HLA-DQB102/HLA-DQB103. However, due to the complexity of the pathogenesis of bipolar disorder, it is unclear whether this gene has a causal relationship with suicidal ideation in patients with bipolar disorder. This may be due to poor sleep and poor diet during the depressive phase of bipolar disorder, which leads to compromised immune function. However, the results of this study warrant further investigation.

Freud believed that the goal of all life is death and proposed the theory of the “death instinct,” which points internally to self-

mutilation or suicide and externally to aggression. Many people with BD are more likely to commit suicide, self-injury, violence, or crime than the general population. In the first part of the experiment, we screened 10 paediatric patients with bipolar disorder for genome-wide DNA methylation differences and found that the methylation levels of genes such as ABI3BP, HLA-DQB1, HLA-DRB1, AUTS2, SP3, NINJ2, and DPYSL2 might be associated with suicidal ideation. Due to the failure of PCR amplification of some gene sequences and limited experimental funding, we selected two genes, ABI3BP and DPYSL2, for pyrophosphate sequencing. ABI3BP, an ABI family member 3 binding protein, has a relatively unknown function that promotes the differentiation of mesenchymal stem cells (MSCs) while inhibiting MSC proliferation [51]. ABI3BP may also play a role in apoptosis and senescence [52]. Moreover, it reduces mitral cell and dendritic cell complexity, a process that is important in brain development because functional circuits are established by pruning immature connections [53]. DPYSL2 is involved in cytoskeletal formation and is closely related to neuronal development. It is also associated with several neurological diseases [54]. In 2020, researchers screened differentially methylated genes after spinal cord injury and sciatic nerve injury using whole-genome bisulphite sequencing and immunoprecipitation sequencing of methylated DNA. A total of 16 genes underwent the same treatment, including DPYSL2 [20]. DPYSL2 plays an important role in axon formation, and DPYSL2 dysfunction may lead to abnormal neurodevelopment [55]. The results of this experimental study found no significant correlation between the ABI3BP or DPYSL2 gene and the medium-to-high risk of suicide when comparing the low- and medium-to-high-risk groups ($P > 0.05$). However, Kimbrel et al. [56] observed that multiple variants of ABI3BP were associated with suicidal behaviours. One investigator performed a genome-wide screening of whole blood from approximately 2900 patients with major depressive disorder and approximately 5800 patients with bipolar disorder and found an association between ABI3BP and suicide attempts in patients with major depressive disorder [57]. Lee et al. [58] found that two functional SNPs in DPYSL2 (promoter region rs9886448 and exon region rs2289593) were associated with schizophrenia. Nakata et al. [59] found that polymorphisms at the 3' end of the DPYSL2 gene were associated with schizophrenia, especially paranoid schizophrenia, in a Japanese population. Johnston-Wilson et al. [60] found that patients with schizophrenia, bipolar disorder, and major depressive disorder have significantly lower levels of frontal cortical DPYSL2 protein.

As mentioned earlier, the differentially methylated genes in paediatric patients with bipolar disorder who had suicidal ideation and those who did not have were associated with multiple immune-related signalling pathways in pathway analysis. In the GO analysis, we also found that almost all of the top five signalling pathways involved in differentially methylated loci were immune-related. In addition, several studies found that psychiatric disorders, such as bipolar disorder and depression, are associated with the immune system. ABI3BP has been reported to be associated with many immune cell marker sets in many types of cancer. Yan et al. discovered that this gene set is related to biological processes such as apoptosis, cell cycle, and DNA damage and that immune infiltration of tumour cells is associated with ABI3BP expression [61]. They also found that the increased expression of ABI3BP in tumours may interfere with the tumour immune microenvironment by affecting immune cell infiltration. In addition, ABI3BP is involved in regulating several immune signalling molecules in lung tumours, including immunostimulants, immunosuppressants, MHC, chemokines, and receptors [62]. Wu et al. [63] found a correlation between DPYSL2 expression and immune cell infiltration in lung adenocarcinomas, including macrophages, dendritic cells, $CD4^+$ T cells, and neutrophils. Moreover, in Gene Set Enrichment Analysis, six immune-related signalling pathways were associated with DPYSL2. In this study, due to the large difference in sample size between the low- and medium-to-high risk groups, the small sample size, and the fact that experimental errors cannot be excluded, which may lead to false-negative results, it cannot be concluded that methylation of the ABI3BP and DPYSL2 genes is not associated with a medium-to-high risk of suicide in paediatric patients with bipolar disorder. Therefore, this study requires further exploration and validation with a larger sample size.

The limitations of this study are as follows: (1) No controlled study was conducted before or after drug treatment. (2) The sample size is small, and the reliability of the results needs to be verified by expanding the sample at a later stage. (3) Due to research difficulties, such as difficulty in collecting samples from paediatric patients, a healthy control group was not established in this study. Differences in gene methylation between paediatric patients with bipolar disorder and the healthy population were not explored. (4) There were more confounding factors in the study sample and more heterogeneity between samples. Further research on the correlation between environmental factors and suicide is needed.

5. Conclusion

We used Illumina HD 850K microarray technology to screen genome-wide differentially methylated genes in 10 PBD patients with and without suicidal ideation. After screening genes associated with suicide in PBD (ABI3BP gene, DPYSL2 gene), we hypothesized that the severity of suicide risk in PBD patients correlates with the degree of methylation of the screened genes. We used pyrophosphate sequencing to sequence 45 patients with PBD. These results suggest that suicidal ideation is correlated with methylation levels of differentially methylated genes in pediatric bipolar disorder. However, the severity of suicide risk in pediatric bipolar disorder patients may not be correlated with the degree of methylation of the ABI3BP and DPYSL2 genes. Further validation is still needed.

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Data availability statement

The data are not publicly availiable due to privacy or ethical restrictions.The authors do not have permission to share data.

CRediT authorship contribution statement

Dilinazi Kari: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Peierdun Mijiti:** Formal analysis, Writing – review & editing. **Shaohong Zou:** Funding acquisition, Methodology, Writing – review & editing. **Peiwen Zhang:** Data curation, Investigation.

Declaration of competing interest

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