

A Pilot Study on DNA Methylation in Pediatric HAdV-7-induced Sepsis

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Research Article

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Abstract

Human adenovirus (HAdV) is one of the most common respiratory pathogens affecting children. HAdV infection has high morbidity and mortality, and it may lead to severe complications and long-term pulmonary sequelae. However, the pathogenesis of pediatric HAdV-7-induced sepsis remains unclear. The analysis of DNA methylation profiles in peripheral blood is attracting increasing attention as an effective method for investigating the pathogenesis of various diseases and identifying biomarkers of disease progression. Here, we performed reduced representation bisulfite sequencing to analyze DNA methylation in peripheral blood samples collected from 11 children with HAdV-7-induced sepsis and 5 healthy children. The Metilene software was used to analyze differential methylation in the two groups. We also performed functional enrichment analysis of the genes with differentially methylated regions (DMRs). We detected 1,138 DMRs between the two groups. Additionally, 122 DMRs were detected between the HAdV-7-induced sepsis survivor and non-survivor groups. After screening based on biological and clinical significance, we found that a group of genes (*KCNQ10T1*, *KPNB1*, *GRB10*, *HOXA5*, *HOXA4*, and *BCL9L*) with differential methylation played an essential role in Wnt signaling. Additionally, genes related to the Wnt/ β -catenin signaling pathway, such as *MEG3*, *GNAS-AS1*, and *GNAS*, exhibited differential methylation in the survivor and non-survivor groups. Our data suggest that specific patterns of DNA methylation are associated with the occurrence and progression of HAdV-7-induced sepsis. Wnt signaling was also affected by the changes in methylation. Thus, we identified potential biomarkers and therapeutic targets for pediatric HAdV-7-induced sepsis.

1. Introduction

Human adenovirus (HAdV), a non-enveloped virus with a linear double-stranded DNA genome, belongs to the genus *Mastadenovirus* and family *Adenoviridae* [1]. HAdV infections account for at least 5–10% of all respiratory infections in children [2]. Human adenovirus 7 (HAdV-7) has been implicated in epidemics of severe lower respiratory tract infections, and it is among the viruses that are the most strongly associated with respiratory disease outbreaks in children aged less than 5 years worldwide [2]. HAdV-7 infection is usually mild and self-limiting in most immunocompetent patients [3]. However, the incidence of HAdV-7-related severe or life-threatening infection in children is increasing. Further, severe adenoviral infections in children can be complicated by acute respiratory distress syndrome (ARDS), respiratory failure, and sepsis [2, 4].

Although the role of HAdV-7-induced sepsis in lung injury remains poorly understood, it is considered to be related to immune system activation in the host [5]. In particular, the innate immune response is considered to contribute significantly to lung tissue damage caused by HAdV-7-induced sepsis [5]. Although the innate immune response induced by HAdVs is well documented, the mechanism underlying sepsis and inflammatory injury following HAdV-7 infection remains poorly understood.

Epigenetic changes, including DNA methylation, histone modification, and RNA regulation, significantly affect gene expression during disease development. DNA methylation is one of the most stable epigenetic changes, and its relevance for prognosis and early diagnosis has been demonstrated in multiple diseases [6–8]. In epigenetic studies, blood is a readily accessible tissue source that frequently exhibits changes in response to diseases and environmental exposure to infectious agents [9]. Although the absolute magnitude of DNA methylation at specific loci differs in different tissues, disease-related interindividual changes in DNA methylation are maintained across tissues within an individual [10]. There is ample evidence linking DNA methylation to immune activation and immune tolerance in humans [11, 12]. However, to date, only a limited number of studies have explored DNA methylation in children with HAdV-7-induced sepsis.

In this study, we aimed to identify characteristic DNA methylation patterns and related signaling pathways in children with HAdV-7-induced sepsis using whole blood samples. The results of this study provide insights into the pathogenesis and progression of HAdV-7-induced sepsis and may help identify more effective therapeutic targets.

2. Materials And Methods

2.1 Research objective

This was a retrospective cohort study conducted at the pediatric intensive care unit (PICU) of Guangzhou Women and Children's Medical Center from April 2018 to April 2020. The inclusion criteria for children with HAdV-7-induced sepsis were: (1) age less than 18 years and greater than 30 days; (2) confirmed diagnosis of severe pneumonia; (3) confirmed diagnosis of HAdV-7 infection, based on polymerase chain reaction (PCR) using a throat swab or in-depth sputum sample; (4) a Sequential Organ Failure Assessment score ≥ 5 points, or a Murray Lung Injury Score > 2 points. Eleven children with HAdV-7-induced sepsis were included. During the same period, five healthy children who had undergone physical examination in the same hospital were included as controls. Whole blood samples were collected from the patients at admission to the PICU, while those from healthy children were collected after physical examination. All blood samples were stored at -80°C . The study was approved by the ethics committee of Guangzhou Women and Children's Medical Center (Approval no.: 57301). Informed consent was obtained from the parents of the children before study initiation.

2.2 Data collection

Data on gender, age, clinical diagnosis, and treatment process were collected for each patient with HAdV-7-induced sepsis. However, gene and age data were not collected from the controls.

2.3 Construction of an RRBS library

Genomic DNA was first isolated from the peripheral blood samples using the Qiagen Blood Mini Kit (Qiagen, Hilden, Germany), and then subjected to agarose gel electrophoresis. The details of RRBS library construction have been reported previously [13]. First, $1\ \mu\text{g}$ of DNA was digested using 100 U of *MspI* restriction enzyme (New England BioLabs, Ipswich, MA, USA) at 37°C for 16 h. Methylated adapter ligation was performed following blunt ending and dATP

addition. Adapter-ligated DNA fragments of 160–350 bp, excised from the 2% agarose gels, were used for the amplification of *MspI*-digested DNA by PCR. Bisulfite modification of the extracted DNA was performed using the Zymo EZ DNA Methylation-Gold Kit™ (ZYMO Research, Irvine, CA, USA) according to the manufacturer's instructions. After bisulfite treatment, a methylated cytosine was retained as "C", whereas a non-methylated cytosine was replaced with "U." During PCR amplification, the "U" bases were replaced with "T" bases. PCR amplification for the generation of the final libraries was performed using the JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, USA). The cycling conditions were as follows: 13 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. After the analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and quantification using qPCR, the RRBS libraries were sequenced using the Illumina X Ten platform with 150 bp of paired-end reads.

2.4 Data analysis

After the raw sequencing data were processed using the Illumina base-calling pipeline, reads containing more than 30% "N"s or with low-quality values (< 20) in over 10% of the sequence were eliminated. Concurrently, adapter contamination was eliminated using Cutadapt (version 1.9) [14]. The corresponding annotation files were acquired from the UCSC Genome Database, and the clean reads were aligned to the reference genome using BSMAP (version 2.73) [15]. Only the uniquely aligned sequences containing the *MspI* restriction enzyme digestion site at the end of the read sequences were considered for further analysis. The commonly covered CpG sites with sequencing depths $\geq 5\times$ were selected in each group as candidate sites to conduct global principal component analysis. After bisulfite treatment, the cytosines were read as "T" if unmethylated, or as "C" if methylated, and CpG site methylation was identified. The methylation level of cytosines was defined as the ratio of "C" counts to the total "C" and "T" counts in the sequenced reads. Metilene (version 0.2-7) [16] was used to identify the DMRs between the two groups based on the following criteria: distance between two neighboring candidate CpG sites ≤ 300 bp, ≥ 5 CpG sites, methylation level difference > 0.1 , and Q-value < 0.05 obtained using the Benjamini–Hochberg method. Gene promoters were defined as the regions 2-kb upstream and 0.5-kb downstream of the transcriptional start site. Differentially methylated genes were identified based on the presence of one or more DMRs with $> 50\%$ sequence overlap with the promoter or gene body. The gene set pathway annotation was retrieved from the gene ontology (GO) database (<http://geneontology.org/page/go-database>) [17]. Functional enrichment analysis was performed using the hypergeometric test. Additionally, the Benjamini–Hochberg method was used to control the false discovery rate (FDR) with a threshold of 0.05.

2.5 DMR selection

The DMRs located in the promoter regions were sorted based on low to high FDR. The 20 DMRs with the lowest FDR were selected for the analysis.

3. Results

3.1 Patient characteristics

Table 1 summarizes the clinical characteristics of the 11 pediatric patients with HAdV-7-induced sepsis, which included five deceased patients. The mean age was 22.3 ± 25.9 months, and the mean weight was 10.8 ± 2.9 kg. Females accounted for 36.4% of the patient population. All patients were on ventilator support and received vasoactive drugs, 63.6% received extracorporeal membrane oxygenation, and 54.5% received high-frequency ventilation. The mean duration of hospitalization was 27 ± 14 days, and the duration of PICU stay was 21 ± 15 days. The median length of stay in the hospital before admission to the PICU was 7 days. The mean PaO₂/FiO₂ (P/F) index was 74.2 ± 30.9 . In patients with HAdV-7-induced sepsis, 45.5% mortality was recorded. The reduction of the P/F index (< 150) indicated that all patients suffered from ARDS and sepsis.

3.2 Methylation levels

3.2.1 Regions and genes with differential methylation

The DNA methylation level in blood was compared between patients with HAdV-7-induced sepsis and healthy controls as well as between surviving and non-surviving patients with HAdV-7-induced sepsis (Table 2). A total of 1,138 DMRs were identified following FDR correction for multiple testing. Among these, 241 DMRs showed hypomethylation, and 897 showed hypermethylation between the HAdV7-induced sepsis group and the control group. Moreover, 122 DMRs were identified between the HAdV7-induced sepsis survivor and non-survivor groups, with 89 DMRs being hypomethylated and 33 being hypermethylated.

3.2.2 Location of CpG regions with differential methylation

A total of 1,138 DMRs were detected between patients with HAdV-7-induced sepsis and controls (Fig. 1). Among these, 41 (17.01%) hypermethylated and 163 (18.17%) hypomethylated DMRs were located in the promoter regions of the genes. Additionally, 122 DMRs were detected between the survivor and non-survivor groups, and among these, 22 (24.72%) hypermethylated and 13 (39.39%) hypomethylated DMRs were located in the promoter regions (Table 3).

3.2.3 Chromosomal distribution in regions with differential methylation

The chromosomal distribution of DMRs was further analyzed. The DMRs that distinguished patients with HAdV-induced sepsis from healthy controls were primarily located on chromosomes 19, 1, 17, and 2. The DMRs between surviving and non-surviving patients with sepsis were primarily located on chromosomes 19, 20, 17, and 9 (Table 4).

3.3 GO enrichment analysis

The comparison between controls and patients with HAdV-7-induced sepsis revealed that genes associated with the $\alpha\beta$ T cell receptor complex, apoptosis, and nitrogen compound metabolism contained regions of hypermethylation (Fig. 2a), whereas those associated with serotonin secretion from platelets, signal transduction, and creatine kinase activity contained regions of hypomethylation (Fig. 2b). The comparison between the survivor and non-survivor groups

showed that genes associated with autophagy and endosomal transport contained hypermethylated regions (Fig. 2c), whereas those associated with the cell cycle, p53 binding, p53-mediated signal transduction, and nucleosomes contained hypomethylated regions (Fig. 2d).

3.4 Key site screening

The DMRs in the promoters between the HAdV-7-induced sepsis and control groups were sorted based on low to high FDR, and the 20 DMRs with lowest FDR were selected for analysis. Among the 44 genes corresponding to the selected DMRs, 6 (13.6%) (*CD3D*, *IRGM*, *CD3/CD3G*, *KPNB1*, *HELZ2*, and *TNFRSF6B*) were found to encode sepsis-related proteins. The GenBank database (<https://www.ncbi.nlm.nih.gov/>) was referred to for gene function information. The expression of the selected DMRs correlated with that of *KCNQ1OT1*, *KPNB1*, *GRB10*, *HOXA5*, *HOXA4*, and *BCL9L*.

The DMRs in the promoters between the HAdV-7-induced sepsis survivor and non-survivor groups were also sorted based on low to high FDR, and 20 DMRs with the lowest FDR were selected for analysis. The expression of the selected DMRs correlated with that of *MEG3*, *KCNQ1OT1*, *HOXA4*, *GNAS-AS1*, and *GNAS*.

4. Discussion

To our knowledge, this study is the first to investigate epigenome-wide DNA methylation in children with HAdV-7-induced sepsis and healthy control children. The DNA methylation status in surviving and non-surviving patients was also compared. Our analysis of 11 children with HAdV-7-induced sepsis and 5 healthy controls led to the identification of 1,138 DMRs related to HAdV-7-sepsis occurrence and 122 DMRs related to sepsis progression. The functional enrichment analysis of 239 genes with DMRs located in the promoter region indicated the involvement of these genes in apoptosis, T cell activation, serotonin secretion from platelets, and autophagy. Since abnormalities in these functions have been reported in sepsis [18–21], our data are reliable.

In this study, 20 DMRs located in gene promoters and selected based on the FDR value could be used to distinguish pediatric patients with HAdV-7-induced sepsis from healthy controls and surviving patients from non-surviving patients. Of the affected genes, multiple genes, including *KCNQ1OT1*, *KPNB1*, *GRB10*, *HOXA5*, *HOXA4*, *BCL9L*, *MEG3*, *GNAS-AS1*, and *GNAS*, were associated with the Wnt/ β -catenin signaling pathway. The Wnt/ β -catenin signaling pathway plays a vital role in cell migration, genetic stability, and apoptosis, and it has also been linked to developmental diseases and cancer. As Wnt/ β -catenin signaling affects many other signaling cascades, its dysfunction may lead to deleterious effects. A previous single-center study on three neonates with sepsis and three healthy neonates [22] and an analysis on 134 patients [23] (68 patients of sepsis and 66 controls) reported that genes associated with the Wnt/ β -catenin signaling pathway undergo methylation changes in response to sepsis. However, these studies reported different sepsis-inducing pathogens. Our findings suggest that the Wnt/ β -catenin signaling pathway plays an essential role in the pathogenesis and progression of HAdV-7-induced sepsis.

The relationship between viral infection and the Wnt/ β -catenin signaling pathway has been described previously. Currently, increasing evidence has shown the importance of this pathway in the replication, latency, and pathogenicity of multiple viruses, including human immunodeficiency virus [24], hepatitis C virus [25], and cytomegalovirus [26]. However, to date, only limited information has been published on the effect of HAdV-7 infection on Wnt/ β -catenin signaling. Our study is the first to present evidence on this association. We found that the levels of methylation in *KCNQ1OT1*, *KPNB1*, and *GRB10* were higher in children. In contrast, the levels of methylation in *HOXA5*, *HOXA4*, and *BCL9L* were lower in patients than in controls.

The multiple differentially methylated genes identified in our study share established functional relationships with the Wnt/ β -catenin pathway. The long non-coding RNA of *KCNQ1OT1* directly interacts with β -catenin, inhibits its degradation, and upregulates Wnt/ β -catenin signaling [27]. *KPNB1* encodes a transport factor in nuclear membrane that might assist β -catenin transport across the nuclear membrane via Wnt signaling [28]. *GRB10* is a multi-module adapter protein known to interact with various transmembrane tyrosine kinase receptors and with the low-density lipoprotein receptor, a component of the Wnt cell surface receptor. Wnt-induced phosphorylation of low-density lipoprotein receptor-related protein 6 might influence the degradation of β -catenin, which is a key regulatory step in the Wnt pathway [29]. *HOXA5* is a critical regulator of stem cell differentiation; it contributes the development of the respiratory system and inhibits Wnt/ β -catenin signaling [30]. *HOXA4* is a transcription factor that inhibits Wnt signaling by suppressing the protein expression of β -catenin, cyclin D1, c-Myc, and surviving [31]. *BCL9L* is a component of the β -catenin degradation complex, and the activity of this complex is inhibited by the activation of Wnt signaling [32]. We found that the methylation level of *GNAS-AS1* was higher in surviving than in non-surviving patients with HAdV-7-induced sepsis. Conversely, the methylation levels of *MEG3*, *KCNQ1OT1*, and *HOXA4* were lower in survivors than in non-survivors. Previous studies have shown that *MEG3* interacts with *miR4261* to relieve the inhibition of Dickkopf-2 and block Wnt/ β -catenin signal transduction [33]. *GNAS-AS1* regulates Wnt/ β -catenin signaling by mediating β -catenin expression [34]. *GNAS* is a critical component of the cell membrane receptor pathway and can activate Wnt/ β -catenin signaling [35]. However, in this study, *GNAS* was found to contain both hypermethylated and hypomethylated regions. While further studies are required to confirm the expression level of *GNAS* in response to sepsis, adequate literature on this topic remains unavailable. Our findings suggest that the regulation of Wnt/ β -catenin signaling may play a role in the occurrence and development of sepsis caused by HAdV-7 in children.

5. Conclusions

In this study, we used RRBS to evaluate the abnormal DNA methylation patterns in whole blood samples from children with HAdV-7-induced sepsis. Our findings indicate that the changes in the DNA methylation patterns in peripheral blood cells are related to the pathogenesis and progression of HAdV-7-induced sepsis. In particular, genes associated with Wnt/ β -catenin signaling and the epigenetic changes in these genes may play a role in this disease.

Our study has some limitations. First, we used whole blood samples, with the proportion of immune cells differing between samples. Therefore, our analysis may be more informative with respect to specific immune cell alterations. Second, the relationship between DNA methylation and gene expression levels was not explored. Third, the sample size was small, and the results should be confirmed in studies with a larger sample size as well as in studies with animal

models and gene knockout experiments. Nevertheless, our findings provide evidence that the analysis of DNA methylation is a suitable method for evaluating the pathogenesis of pediatric HAdV-7-induced sepsis and may help develop new prognostic and therapeutic tools.

Declarations

Funding

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Conflicts of interest

None.

Availability of data and material

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

Code availability

Not applicable

Authors' contributions

Bolun Huang, Feiyan Chen, Run Dang, Wenmin Yang, Hongyan Peng, Chunmin Zhang, Yunlong Zuo, Jie Hong, Mingqi Zhao, Yi Chen and Yiyu Yang contributed to the study conception and design. Material preparation, data collection and analysis were performed by Feiyan Chen and Bolun Huang. The first draft of the manuscript was written by Bolun Huang, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

The study was approved by the ethics committee of Guangzhou Women and Children's Medical Center (Approval no.: 57301).

Consent to participate

Written informed consent was obtained from the parents or guardians of the participants.

Consent for publication

Not applicable.

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Tables

Number	Gender	Age (months)	Weight at admission (kg)	Duration of illness before admission (days)	Hospitalization duration (days)	Length of PICU stay (days)	Number of days of invasive ventilation	High frequency ventilation	ECMO ^a	Prognosis	P/F ^b	W
1	Female	17	10	15	25	24	25	Y	N	Death	72	1
2	Male	7	10	10	7	7	7	N	Y	Death	36	7
3	Male	36	16	7	57	57	57	Y	Y	Death	66	2
4	Male	8	8	25	5	5	5	N	Y	Death	36	9
5	Male	10	9	7	24	22	22	Y	Y	Death	81	6
6	Male	6	8	5	39	13	11	Y	Y	Improvement	93	1
7	Male	96	17	15	28	21	18	Y	Y	Improvement	43	5
8	Female	14	10	7	38	38	38	Y	Y	Improvement	60	6
9	Female	12	10	7	27	12	8	N	N	Improvement	97	2
10	Female	20	12	14	27	18	15	N	N	Improvement	94	1
11	Male	19	10	7	23	11	11	N	N	Improvement	138	2

^a ECMO: Extracorporeal Membrane Oxygenation.

^b P/F: PaO₂/FiO₂, oxygenation index.

Pair	nDMR	nCG	length	nDMR_hyper	nCG_hyper	length_hyper	nDMR_hypo	nCG_hypo	length_hypo
HAdV7-induced sepsis vs. control	1138	11306	233558	241	2438	47940	897	8868	185618
Surviving vs. non-surviving patients with HAdV7-induced sepsis	122	1992	24222	89	1563	18797	33	429	5425

Table 3 Locations of CpG regions with differential methylation					
Surviving vs. non-surviving patients with HAdV-7-induced sepsis				Mild vs. Severe patients with HAdV-7-induced sepsis	
element	variation	count	rate	count	rate
promoter	hyper	41	0.1701	22	0.2472
	hypo	163	0.1817	13	0.3939
5' UTR	hyper	5	0.0207	8	0.0899
	hypo	28	0.0312	1	0.0303
exon	hyper	61	0.2531	45	0.5056
	hypo	191	0.2129	9	0.2727
intron	hyper	126	0.5228	32	0.3596
	hypo	471	0.5251	17	0.5152
3' UTR	hyper	4	0.0166	3	0.0337
	hypo	38	0.0424	1	0.0303
Intergenic region	hyper	92	0.3817	35	0.3933
	hypo	386	0.4303	12	0.3636

Table 4 Chromosomal distribution of regions with differential methylation						
chr	Patients with HAdV-7-induced sepsis vs. healthy controls			Surviving vs. non-surviving patients with HAdV-7-induced sepsis		
	hyper	hypo	Total	hyper	hypo	Total
chr1	20	76	96	6	2	8
chr2	16	56	72	2	5	7
chr3	12	27	39	3	1	4
chr4	12	23	35	2	0	2
chr5	10	30	40	4	0	4
chr6	6	28	34	3	0	3
chr7	18	48	66	3	3	6
chr8	8	39	47	3	0	3
chr9	10	53	63	6	2	8
chr10	9	39	48	1	2	3
chr11	11	49	60	3	1	4
chr12	12	41	53	1	2	3
chr13	4	12	16	2	0	2
chr14	11	17	28	2	1	3
chr15	3	11	14	1	2	3
chr16	12	56	68	5	2	7
chr17	19	75	94	9	2	11
chr18	4	23	27	1	1	2
chr19	30	93	123	15	1	16
chr20	5	31	36	7	4	11
chr21	4	18	22	1	0	1
chr22	4	39	43	4	1	5
chrX	1	7	8	1	1	2
chrY	0	0	0	0	0	0
chrM	0	6	6	0	0	0

Figures

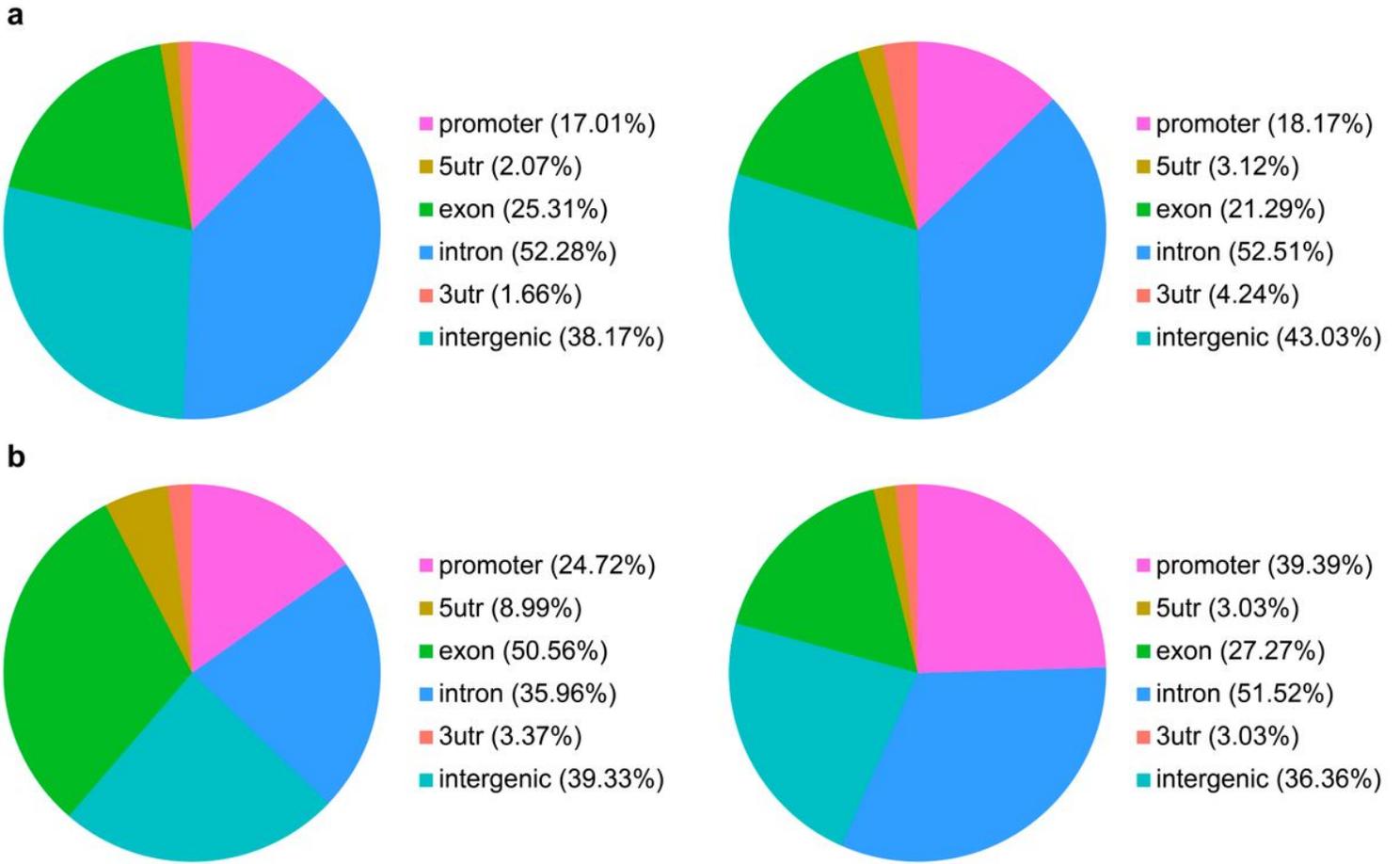


Figure 1
 Pie chart representing the CpG regions with differential methylation between the study groups. (a) Patients with HAdV-7-induced sepsis vs. healthy controls. (b) HAdV-7-induced sepsis groups: survivors vs. non-survivors. CpG, cytosine-phosphate-guanine; UTR, untranslated region.

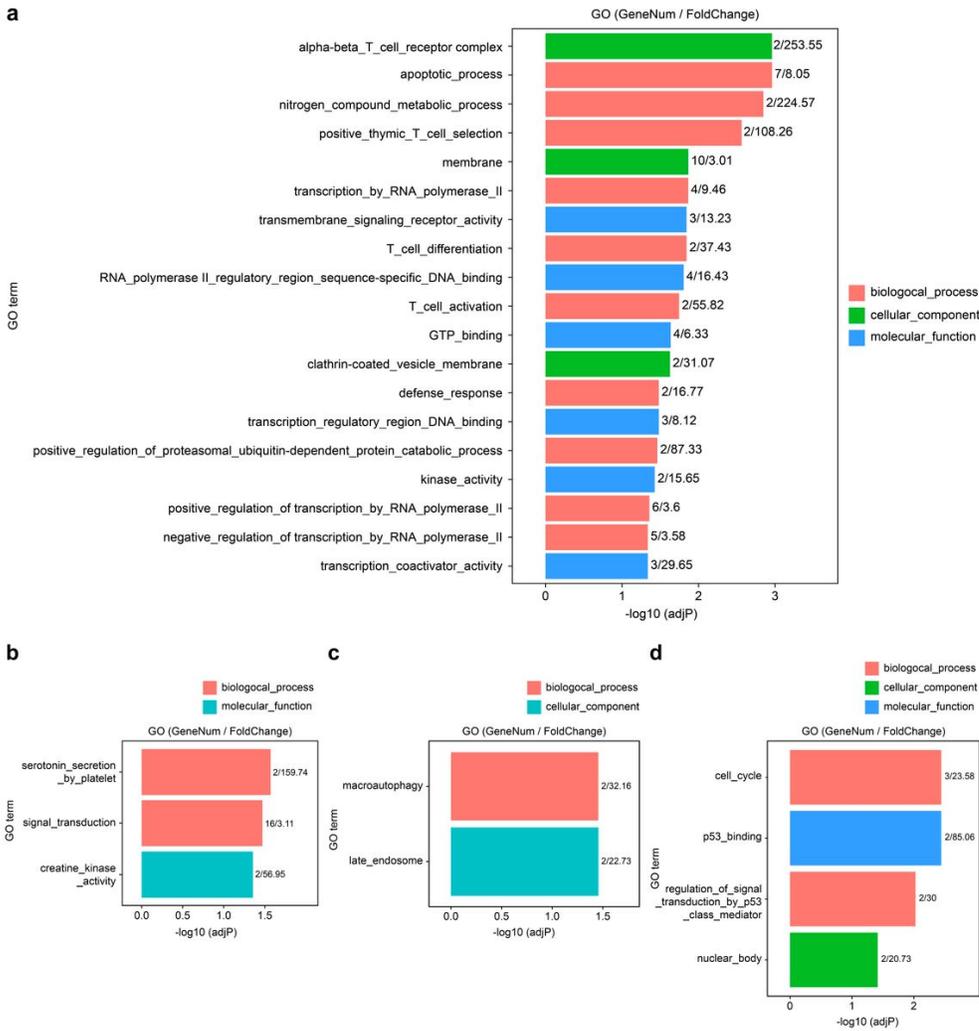


Figure 2

Frequency profiles of second-level entries based on gene ontology enrichment analysis. (a) Gene ontology analysis between patients with HAdV-7-induced sepsis and healthy controls (genes with hypermethylation). (b) Gene ontology analysis between patients with HAdV-7-induced sepsis and healthy controls (genes with hypomethylation). (c) Gene ontology analysis between the HAdV-7-induced sepsis survivor and non-survivor groups (genes with hypermethylation). (d) Gene ontology analysis between the HAdV-7-induced sepsis survivor and non-survivor groups (genes with hypomethylation)