Implication of m6A methylation regulators for the immune microenvironment of bronchopulmonary dysplasia

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Research Article

Keywords: Bronchopulmonary dysplasia; bioinformatics, m6A RNA methylation regulators, epigenetic modifications

Posted Date: September 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2046982/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Biochemical Genetics on February 23rd, 2024. See the published version at https://doi.org/10.1007/s10528-024-10664-1.
Abstract

**Objective:** to evaluate the effect of N6-methyladenosine (m6A) RNA methylation regulators on the development of bronchopulmonary dysplasia (BPD).

**Methods:** Transcriptome data related BPD was downloaded from the GEO. Differentially expressed m6A methylation regulators between BPD and control group were identified. Consensus clustering was conducted for the classification of BPD and its association with the phenotypes were conducted. Differentially expressed genes (DEGs) and immune related DEGs (DEMGs) analysis was performed. The GSEA, GO and KEGG were applied to interpret the functional enrichments. The composition of immune cell subtypes in BPD subsets was predicted by CIBERSORT analysis.

**Results:** Compared with control group, the alteration of most m6A regulators expression were detected, especially for IGF2BP1/2/3. The BPD was classified into 2 subsets, of which cluster 1 was correlated with severe BPD. Furthermore, the functional enrichment results showed a disturbed immune-related signaling pathway. The CIBERSORT analysis found that the proportion of immune cell subsets changed between cluster 1 and cluster 2.

**Conclusions:** Our study revealed an implication of m6A methylation regulators for the development of BPD, which might provide a novel insight for the diagnosis and treatment for BPD.

Introduction

Bronchopulmonary dysplasia (BPD), the most common complication of prematurity, affects approximately 45% of infants born at less than 29 weeks of gestational age[1]. The great advances in the field of neonatal care contributed to the decline in neonatal mortality rates and improved survival but increased incidence of BPD[2]. BPD is characterized by impaired alveolarization and abnormal vascularization in pulmonary[3]. However, there are long-standing implications for adult health and quality of life associated with BPD, which is not only a lung disease but also a systemic condition as well[4]. In addition, BPD is highly associated with elevated respiratory morbidity and neurodevelopmental impairment.

Currently, it is thought that BPD is caused by a combination of predisposing genetic and environmental factor. Exposed to hyperoxia, maternal infection/inflammation and mechanical ventilation-mediated lung injury are function as environmental risk factors for BPD[5]. Recent researches have confirmed that the development of BPD was accompanied by multiple epigenetic modifications, including chromatin remodeling, histone modification, DNA methylation and noncoding RNA regulation. It has been documented that m6A modification is essential for the development of lung function development[6]. However, the effect of N6-methyladenosine (m6A) RNA methylation on the development of BPD remains undefined.
With the development of multi-omics (including genomics, transcriptomics, proteomics, metabolomics and microbiomics), identification of risk markers for BPD diagnostic becomes to be considerable. Hence, we aimed to evaluate the effect of m6A RNA methylation regulators on the development of BPD, which might provide a novel insight for the diagnosis and treatment of BPD.

**Methods**

**Data collection**

Transcriptome data related BPD was retrieved and downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). Sample size less than 100 was exclude. The GSE32472 includes 182 BPD samples and 112 health control.

**Identified differentially expressed m6A related regulators between BPD and control**

A total of 23 m6A RNA methylation regulators were selected from the published research[7]. The methyltransferase like 3/14 (METTL3/14), WT1-associated protein (WTAP), Vir like m6A methyltransferase associated VIRMA (VIRMA/KIAA1429), RNA-binding motif protein15/15B (RBM15/15B), zinc finger CCCH domain-containing protein 13 (ZC3H13) and Cbl ProtoOncogene Like 1 (CBLL1) were identified as m6A “writer”. Both Fat mass and obesity-associated (FTO) and fat mass alkB homolog 5 (ALKBH5) function as a m6A “eraser” to demethylate the m6A. Thirteen m6A “reader” were included, including YT521-B homology (YTH) domain-containing proteins 1/2 (YTHDC1/2), YTH N6-methyladenosine RNA-binding proteins 1/2/3 (YTHDF1/2/3), heterogeneous nuclear ribonucleoprotein (HNRNP) protein families (including HNRNPA2B1 and HNRNPC) and insulin like growth factor 2 mRNA binding protein families 1/2/3 (IGF2BP1/2/3), FMR1, ELAV-like RNA binding protein 1(ELAVL1) and leucine-rich pentatricopeptide repeat containing (LRPPRC). To further identify the differentially expressed of m6A RNA methylation regulators between BPD and control group, the normalized genes profiles between BPD and control group were compared with Wilcoxon rank sum test. The P value less than 0.05 was considered as a significant difference.

**Consensus clustering for BPD classification and its association with BPD phenotypes**

Unsupervised clustering was conducted to classify the BPD based on m6A RNA methylation regulators signature with R package (Consensus Cluster Plus). Consensus clustering, one of the unsupervised clusters, consists of partition generation and consensus generation based on cooccurrence. To determine the optimal number of clusters k, the cumulative distribution function (CDF) was conducted and its area under the curve was evaluated. In addition, Delta area was depicted to compare the change under the curve between k and k-1. Tracking plot was used to test the robust of clustering. To evaluate the association between cluster classification in BPD and BPD phenotypes, the incidence of severe BPD between cluster 1 and cluster 2 was compared with chi-squared test.
Identification of differentially expressed genes (DEGs) and immune-related DEGs in BPD subsets

To further identify the differentially expressed genes (DEGs) in BPD subsets (cluster 1 and cluster 2) based on m6A methylation signature, the gene expression matrix was compared with linear models for microarray data (limma) analysis. The P value less than 0.05 and |fold change| greater than 1.5 were considered differentially expressed. Subsequently, the differentially expressed immune related genes (DEMGs) were selected through intersecting the DEGs in BPD subsets and immune related genes. The immune related genes were retrieved from the ImmPort database[8]. Pearson correlation analysis was conducted between the differentially expressed lncRNA and 20 m6A methylation regulators.

Function enrichment in BPD subsets

Gene Set Enrichment Analysis (GSEA) [9] was introduced to identify a priori defined set of genes between cluster 1 and cluster2. The dataset of c2.cp.kegg.v7.4.symbols.gmt was downloaded from the Molecular Signatures Database[10]. Gene ontology (GO) enrichment analysis consists of 3 components, including molecular function, cellular component, and biological process. GO analysis[11]. Kyoto Encyclopedia of Genes and Genomes (KEGG) database was widely used to accounting for multi omics studies. Both GO and KEGG enrichment analysis interpret the potential biological phenomena via high-throughput data. The GO and KEGG (ibersort.stanford.edu) database were applied to identify the functional enriched of DEMGs.

CIBERSORT analysis

To investigate the effect of m6A RNA methylation on the immune microenvironment of BPD, CIBERSORT (https://cibersort.stanford.edu/) was applied to estimate the 22 infiltrated immune cells based on genes expression profiles of BPD[12].

Results

Identification of differentially expressed of m6A RNA methylation regulators between BPD and control group

After normalization, only 20 m6A RNA methylation regulators were included for further analysis. There was a clear alteration in the expression of most m6A RNA methylation regulators between the BPD group and control group (Figure 1A). Compared with control group, the expression of YTHDF1, YTHDF2, ZC3H13, FTO, ELAVL1, LRPPRC, RBM15B, METTL14, CBLL1 and FMR1 were down-regulated in BPD group, while the levels of IGF2BP1, IGF2BP2 and IGF2BP3 were up-regulated (P < 0.05) (Figure 1B).

Consensus clustering analysis for BPD classification

Consensus clustering was performed to further investigate the effect of m6A RNA methylation on the clinical heterogeneity of BPD. The heatmap (Figure 2A) and the area under curve for CDF (Figure 2B) identified a clustering for k=2. In addition, the Delta area (Figure 2C) and the tracking plot (Figure 2D)
confirmed a robust clustering (k=2). The BPD samples were clustered into 2 clusters: cluster 1 (n=93) and cluster 2 (n=89).

**Differentially expressed gene and GSEA analysis in BPD subsets**

Compared with cluster 2, there were 102 up-regulated DEGs and 529 down-regulated DEGs in cluster 1 (P<0.05) (Figure 3A). Seven IncRNAs and 521 protein-coding mRNAs were included in DEGs. The GSEA result showed that the interaction between cytokine and cytokine receptor, hedgehog signaling pathway, basal cell carcinoma and maturity onset diabetes of the young were concentrated in cluster 1 (Figure 3B). In addition, the severe BPD (30%) phenotype was correlated with cluster 1 compared with cluster 2 (P<0.05) (Figure 3C).

**Pearson correlation analysis between differentially expressed IncRNA and 20 m6A methylation regulators.**

The results of the Pearson's correlation analysis showed that there was no significant correlation between differentially expressed IncRNAs and 20 m6A methylation regulators. However, compared with other m6A methylation regulators, IGF2BP1 was related to the 7 lncRNAs (Figure 3D).

**Identified the functional enrichment of DEMGs in BPD subsets.**

Focal adhesion, ErbB signaling pathway, T cell receptor signaling pathway, fluid shear stress and atherosclerosis, human T-cell leukemia virus I infection, regulation of actin cytoskeleton, and Epstein-Barr virus infection were enriched in KEGG analysis (Figure 4A). In addition, the humoral immune response, leukocyte migration and neutrophil activation were the main biological processes enriched by GO analysis (Figure 4B). The secretory granule lumen, the cytoplasmic vesicle lumen and the vesicle lumen were related cellular component (Figure 4C). Molecular function of DEMGs concentrated in cytokine binding, receptor regulator activity and receptor ligand activity (Figure 4D).

**Identification of infiltrated immune cell subtypes in BPD.**

Both neutrophils and monocytes were highly enriched in BPD (Figure 5A). Lower proportion of naïve B cells, resting memory T cells and M2 macrophages was observed in cluster 1, while higher percentage of memory B cells, Treg cells and M0 macrophages was detected in cluster 2 (P<0.05) (Figure 5B).

**Discussion**

The subtypes of BPD are poorly defined on account of its substantial heterogeneity in clinical manifestation and outcomes [1, 13]. Frequent overlap in phenotype among different pathologies in severe BPD led to diagnostic confusion [13]. It was suggested that the BPD could be classified into phenotypic subgroups, which might help clinical risk stratification and prognosis prediction [13].
The BPD subset of cluster 1 based on the m6A methylation regulators signature was closely related to the severe BPD, indicating that the m6A methylation might play an important role in the progression of BPD.

Epigenetic modification plays an important role in lung remodeling\[14\]. Epigenetics is defined as modification of genome without altering the DNA sequences in response to environmental stimuli\[15\]. BPD is characterized by abnormal chronic remodeling in lung\[14\]. Maternal inflammation and neonatal hyperoxia, crucial environmental factors in BPD, resulted in epigenetic changes in preterm birth with BPD\[16\]. Hyperoxia induced DNA methylation and H3K27 trimethylation in the BPD mouse model by decreasing the expression of RUNX3\[17\]. Recent study confirmed an increased DNA methylation in infants with severe BPD and in BPD mouse\[16\]. In addition, histone modification is associated with regulating the transcription. It was found that the histone modifications, including H3K4me3, H3K27me3, H3K36me2, H3K79me2, and H4K20me3 were suppressed in animal study\[16\]. A total of 275 differentially methylated CpGs, which engaged in lung maturation and hematopoiesis pathways, were identified in BPD from a recent epigenome-wide association study (EWAS)\[18\]. Subsequently, the transcriptome alteration induced by DNA methylations in preterm cord blood leaded to pulmonary disorder in BPD\[18\]. Long noncoding RNA also regulates the development of BPD. Rain was associated with alleviating hyperoxic damage in lung by sponging miR-421 in BPD mouse\[19\]. Similarly, the lncRNA TUG1 was found to suppress the inflammatory response and cell apoptosis by sponging miR-29a-3p in the lung in an animal study \[20\]. Emerging evidence suggests that m6A RNA methylation functions as a key regulator of gene expression and plays important roles in the occurrence and development of various diseases, including cancer, inflammation diseases and pulmonary dysfunctions.

Postnatal environmental factor-disturbed oxygen levels, including hypoxia and hyperoxia, impacted on the expression of m6A methyltransferase and demethylase proteins. The development of pulmonary hypertension (PH) is accompanied by m6A methylation. Continuous low expression of METTL3 was detected in PH rats exposed to hypoxia\[21\].However, there is a sexually dimorphic epigenomic landscape in response to hyperoxia in the injured neonatal lung\[22\]. Exposure to hyperoxia is crucial for the pathogenesis of BPD\[23\]. Hyperoxia induced acute lung injury in BPD mice through regulating miR-34a\[3\]. High level of ubiquitin-specific protease 7 (USP7) was detected in lung tissue of BPD rat with hyperoxia exposure via Wnt signaling pathway\[24\]. Our study here showed clear evidence for the alteration of m6A methylation in BPD.

The m6A RNA methylation was involved in the progression of chronic obstructive pulmonary disease (COPD). It has been revealed that the m6A regulators (including IGF2BP3, FTO, METTL3 and YTHDC2) were highly associated with the occurrence of COPD\[6\]. Environmental stimuli-PM2.5 exposure induced pulmonary microvascular injury via regulating METTL16-mediated m6A modification\[25\]. METTL16 reduced the translation of Sulf2 and Cyth1 by targeting the methylation sites in Sulf2 and Cyth1 in the lung and led to microvascular injury \[25\]. In addition, m6A RNA methylation had an impact on the presence of acute respiratory distress syndrome (ARDS). Increased protein expression of METTL16 and FTO was observed in ARDS mice treated with lipopolysaccharide (LPS), while decreased
expression of YTHDC1, IGFBP3, YTHDF1 and YTHDF3 was identified [26]. However, the specific mechanism on m6A RNA methylation for ARDS need further investigation.

In our study, difference in m6A RNA methylation regulators between BPD and control group was observed in our study. IGF2BP1 and IGF2BP2 was closely associated with the developmental disorders in BPD[18]. The differentially expressed genes analysis in our study highlighted a crucial role of IGF2BP in BPD. IGF2BP1/2/3, a family of m6A “reader”, targets for the mRNA transcripts by recognizing the m6A consensus motifs -“GGAC”, which enhanced the stability, promotes the storage, and facilitated the translation of their target mRNA[27]. The absence of IGF2BPs leads to the downregulation of target genes globally. Functional enrichment for the IGF2BPs-target genes mainly related to the DNA replication, cell cycle, proliferation and cancer-related biological processes and pathways[27]. In contrast to IGF2BP1/2/3, the m6A reader-YTHDF2 reduced the stability of target mRNA and promoted the mRNA decay[28]. YTHDF2 suppressed the Wnt signaling pathway by binding and degrading the mRNA of ccnd1, c-Myc and Axin2, which reduced the proliferation and differentiation of hematopoietic stem cells[28]. The eraser FTO was down-regulated in the BPD group. FTO reduced the level of m6A methylation in human cells by demethylating m6A[29]. The enhanced m6A levels by inhibiting FTO enabled the recruitment of YTHDF1 and facilitated the translation of MYC, leading to tumorigenesis[30].

Functional enrichment for DEMGs between cluster 1 and cluster 2 confirmed an immune disorder in BPD, especially for severe BPD. Immune microenvironment alteration in BPD has been widely discussed in recent years[31]. Interestingly, epigenetics, especially for DNA hypermethylation, is highly correlated with the immune-related signaling pathway[31]. The hub genes in the B cell of lung concentrated in the PI3K-AKT, cell receptor signaling pathways cytokine-cytokine receptor interaction in BPD mouse. Concurrently, the DNA hypermethylation was observed in immune system-relevant genes[31]. Recent research demonstrated that premature infant with severe BPD was featured with decreased level of monocytes (CD64, HLA-DR) at 1 month of life. In addition, reduced expression of CD14 + CD64 + cells was observed at 38–40 weeks after conception[32]. The proportion of B cells and CD4 cells (CD62L) was reduced in BPD infants[33]. The activation and polarization of macrophages play a crucial role in the development of lung diseases. Increased expression of macrophage-related plasma cytokines was observed in BPD patients. Hyperoxia induced M1-like polarization via Krüppel-like factor 4 in vitro. In addition, macrophage affected the proliferation of alveolar epithelial type II cells by activating the IL6 and the STAT3 signaling pathway[34]. Recent study found that immunomodulation of macrophage phenotype by mesenchymal stromal cell exosomes suppressed BPD inflammation in vitro and in vivo, which could be a potential strategy for BPD treatment [35].

Conclusions

Our study revealed an implication of m6A methylation regulators in the development of BPD, especially for severe BPD, which might provide a novel insight for the diagnosis and treatment for BPD.

Declarations
Ethical Approval

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Tianping Bao and Zhaofang Tian conceived the project. Tianping Bao and Haiyan Zhu performed the study, analyzed the data, and wrote the main manuscript text. Mengmeng Ma, Tingting Sun, Jingjing Hu, JingYan Li, Linxia Cao and Huaping Cheng prepared figures 1-5. Zhaofang Tian analyzed the data and critically revised the manuscript.

Funding

This work was financially supported by National Natural Science Foundation of China (81801495) and key projects of Jiangsu Commission of Health (ZDB2020005).

Availability of data and materials

The data used or analyzed during this study are included in this article and available from the corresponding author upon reasonable request.

References


Figures

(A) Heat map of the expression of 20 m6A RNA methylation regulators between BPD and the control group. ***, P value < 0.01; **, P value < 0.05; *, P value < 0.1, the red grid represents upregulated genes; the green grid represents downregulated genes. (B) Violin plot of the differentially expressed gene of 20 m6A RNA methylation regulators between BPD and the control group. ***, P value < 0.01; **, P value < 0.05; *, P value < 0.1, the red plot represents the BPD group; the blue plot represents the control group.

Figure 1

Differentially expressed of 20 m6A RNA methylation regulators between BPD and control group
Figure 2

Consensus clustering for BPD

(A) Heatmap of consensus clustering. (B) Consensus cumulative distribution function (CDF) plot. (C) Delta area plot. (D) Tracking plot
Figure 3

Differentially expressed gene in BPD subsets based on the m6A methylation signature

(A) Heatmap of top 20 differentially expressed gene in BPD subsets; (B) GSEA for DEGs in BPD subsets. (C) Composition of BPD phenotypes between cluster 1 and cluster 2. (D) Pearson correlation analysis between differentially expressed lncRNA and 20 m6A methylation regulators.
Figure 4

Identified the functional enrichment of DEMGs in BPD subsets.

(A) KEGG analysis for the DEGs in BPD subsets. (B) Biological process for GO analysis in BPD subsets. (C) Cellular component for GO analysis in BPD subsets. (D) Molecular function for GO analysis in BPD subsets.
Figure 5

CIBERSORT analysis for immune cell subtypes in BPD

(A) Bar graph of the composition of infiltrated immune cell subtypes in BPD. (B) Violin plot for comparison of infiltrated immune cell subtypes between cluster 1 and cluster 2.