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Original Article

Exploring the molecular mechanisms of pneumonia induced by Streptococcus pneumoniae infected lung epithelial cells through comprehensive network analysis

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Abstract: Background: Pneumonia is one of the common hazards in the respiratory intensive care unit, which often leads to systemic inflammatory response syndrome (SIRS). At present, alveolar epithelial cells are seen as the target cells of pulmonary inflammation, which can regulate the development and prognosis of inflammation. The aim of this study was to elucidate the epithelial specific response during pulmonary infection. Methods: We collected DNA data of three independent mice from the GEO database. They are the epithelial cells of mice lived with Streptococcus pneumoniae, the epithelial cells of mice not infected with Streptococcus pneumoniae and the non-epithelial cells of mice infected with Streptococcus pneumoniae. Through co-expression module analysis, enrichment analysis, and the hypergeometric test, we can predict the ncRNA and transcription factors that regulate the module. Eight functional modules were obtained for co-expression analysis of potential pathogenic genes. Results: Aqp5, Mak6 and other genes were significantly differentially expressed and had an active regulatory role in the dysfunction module, so they were identified as the key genes for the occurrence and development of pneumonia. Next, we found that ncRNA pivot and transcription factors (TFs) pivot (including microRNA-181a-5p, Stat3, Sp3, etc.) significantly regulate dysfunction modules. Therefore, we identified these potential regulators as dysfunctional molecules in the development of pneumonia. In this study, the GEO data of three groups of mice were tested to help reveal the core dysfunction module and potential regulatory factors of pneumonia. Conclusion: The results of the analysis elucidated the potential role of Streptococcus pneumonia infecting epithelial cells in causing pneumonia, improved our understanding of its pathogenesis, and provided a new reference for the further treatment of pneumonia.

Keywords: Pneumonia, streptococcus pneumoniae, co-expression network, enrichment analysis, regulatory factors

Introduction

Pneumonia is one of the common hazards in the respiratory intensive care unit (ICU), which often leads to systemic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndrome (MODS) if further deterioration is not handled in time. The incidence of pneumonia in children under 5 years old was studied from 1985 to 2008. The results showed that the incidence of pneumonia was 0.06-0.27 time per person year, and the mortality rate of pneumonia was 184/100,000-1223/100,000 population [1]. There are many main causes of pneumonia. Due to the process of urbanization, urban PM increases the adhesion of Streptococcus pneumoniae to human airway epithelial cells [2]. The lowest combined negative likelihood ratio was cough (0.30, 0.09-0.96), fever history (0.53, 0.41-0.69) and respiratory rate (0.43, 0.23-0.83) higher than 40 times per minute [3]. Pneumonia is also the third most frequent complication of postoperative complications, and is associated with significant morbidity and mortality. Despite advances in surgical and anesthetic techniques, it is still a frequent cause of post-operative complications [4]. Depending on the research statistics, the incidence of pneumonia after operation was 0.97% in the sample of hospitalized patients (NIS) from 2009 to 2013. The incidence of pneumonia after the operation was the highest in cardiothoracic surgery (3.3%) and urological surgery (1.73%)

[5]. In addition to the above reasons, Streptococcus pneumoniae is another important factor leading to the occurrence and development of pneumonia. The progress of pneumonia requires pathogens to reach the alveoli, and the host's defense is overwhelmed by the toxicity of microorganisms or the size of inoculants, which can be transmitted through carriers, blood sources, etc. [6]. At present, more and more studies have demonstrated that epithelial cells play a special role in pneumonia. Alveolar epithelial cells are traditionally thought to be the target cells of pulmonary inflammation and play a role in regulating the development and prognosis of inflammation [7]. For target cells, studies have shown that the secretion and transmembrane (Sectm) 1 gene - Sectm1a and Sitm1b, in which Sectm1a increases the expression of CXCL2 by neutrophils infected with the lung [8]. In addition to related genes, studies have shown that Streptococcus pneumoniae surface protein K (PspK) increases the binding of Streptococcus pneumoniae to epithelial cells, and enhances the colonization of Streptococcus pneumonia independent of genetic background. Although PspK may contribute to the persistence of non-typed Streptococcus pneumonia (NTSp), it is not associated with invasion [9]. Another protein, Pht, has been proven to play a role in the adhesion of Streptococcus pneumoniae to epithelial cells. Because PhtD antibody can inhibit bacterial adhesion to cells, it suggests that PhtD antibody on the mucosal surface may protect cells from adhesion of Streptococcus pneumoniae and subsequent colonization [10].

Despite the fact that there are more and more studies on Streptococcus pneumoniae infection of lung epithelial cells, the mechanism of its action remains to be studied. The aim of this study was to elucidate the epithelial-specific response during pulmonary infection and to identify the potential role of Streptococcus pneumoniae in the process of infection of lung epithelial cells, so as to further provide more treatment and prevention reference for clinical cases of pneumonia brought about by Streptococcus pneumoniae infection.

Materials and methods

Data resources

Gene Expression Omnibus (GEO) is an international public knowledge base, which can archive and distribute high throughput gene expression and other functional genome datasets free of charge. With the rapid development of sequencing technology, GEO has accepted high-throughput data for numerous other data applications, including genome methylation, chromatin structure and genome-protein interaction data. We downloaded data on GEO database (GSE71623) from infected and uninfected epithelial cells, non-infected epithelial cells and uninfected cells for differential analysis [11].

Difference analysis

Differential expression analysis of gene expression profile data in this study was implemented by R language limma package [12-14]. First, the "Background Correct" function is used to correct and standardize the background of the data. Second, control probes and the low expression probes were filtered out by the quantile normalization method of "Normalize Between Arrays" function.

Co-expression analysis

In order to study the molecular process of epithelial cells in mice infected with Streptococcus pneumoniae, we analyzed the differences between disease samples and normal samples of three groups of independent mice, and integrated the differential gene expression profiles between infected mice and uninfected mice. We used weighted gene co-expression network analysis (WGCNA) [15] to analyze the matrix of differentially expressed genes obtained from the study, and to find the gene module of co-expression. According to the regulation of genes, in each dysfunction module, we excavated the key genes that lead to dysfunction module, and thought that they are the key genes that lead to the occurrence and development of pneumonia.

Enrichment analysis

Exploring the function and signaling pathway of gene involvement is often advantageous to study the molecular mechanism of disease. Enrichment and analysis of the function and pathway of genes of dysfunction module are an effective means to explore the potential mechanism of action of lung epithelial cells infected by Streptococcus pneumonia on pneumonia. Therefore, we analyzed the co-expres-

sion of eight modules between the epithelial cells of mice infected with Streptococcus pneumoniae and those of mice not infected with Streptococcus pneumoniae. The Cluster Profiler package [16] in R language was used to analyze the enrichment of Go function and KEGG pathway. Cluster Profiler is a software package of Bioconductor, which can perform statistical analysis and visualization of functional clustering of gene sets or gene clusters. In addition, we use BinGO [17] applications of Cytoscape to analyze the integrated module network. Through further enrichment analysis of function and pathway of module genes, the functional mechanism of related modules was identified.

Transcription factors and ncRNA of regulatory dysfunction module

Non-coding RNA (ncRNA) and transcription factor (TF) are often the core drivers of gene transcription and post-transcriptional regulation. Therefore, we have scientifically predicted and tested the role of epithelial cell genes infected by Streptococcus pneumoniae in the dysfunction module of pneumonia. Pivotal regulators are defined as regulators that have significant regulatory effects on modules during the progress of pneumonia.

KEGG pathway

KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (https://www.kegg.jp/).

Results

Identification of pneumonia-related disorders

In order to elucidate the molecular changes in the process of epithelial-specific reaction during pulmonary infection, the differential expression of epithelial cells in Streptococcus pneumoniae-infected mice, non-infected mice and non-epithelial cells in Streptococcus pneumoniae-infected mice were analyzed based on microarray data. Differentially expressed genes were integrated to obtain genes that may assist in the development of pneumonia. The difference was taken by using epithelial cell infection and non-infection, non-epithelial cell infection and non-infection respectively. By combining both groups of differential genes, 5069 differential genes were obtained. We think there are pneumonia expression disorders in these differentially expressed genes.

Recognition of pneumonia functional disorder module

Expression profiles of 5069 differentially expressed genes and their interacting genes in pneumonia epithelial cells were constructed. Then, based on weighted gene co-expression network analysis (WGCNA), we observed significant grouping co-expression of these genes in the samples. Modularization is a subsystem that deals with global complex systems and decomposes them into more detailed and orderly subsystems. Each subsystem has its characteristics. Clustering the expression behavior of infected and uninfected epithelial cells, non-infected and uninfected epithelial cells into modules are helpful for us to observe the complex synergistic relationship between these genes from the perspective of expressive behavior. Therefore, by identifying the co-expression group as a module, we obtained eight functional impairment modules (**Figure 1A**, **1B**). The key genes of each module were identified based on functional impairment module, and the core genes with Aqp5, Mak6 and so on were obtained. By associating the modules with the phenotypic data of different treatments, we found that MEturquoise and Ered were significantly positively correlated with the phenotype of pulmonary epithelial cells infected by Streptococcus pneumoniae (**Figure 1C**).

Functions and pathways of modular gene participation

We analyzed the enrichment of GO function and KEGG pathway in 8 modules, and obtained 24087 biological processes, 3021 cell components, 5064 molecular functions and 1016 KEGG pathways. It was noted that these functions mainly focused on the biological processes such as GTPase activity, ATPase activity, methyltransferase activity and catalytic activity (**Figure 2A**). On the other hand, enrichment of KEGG pathway reflects that the differentially expressed genes in infected and uninfected

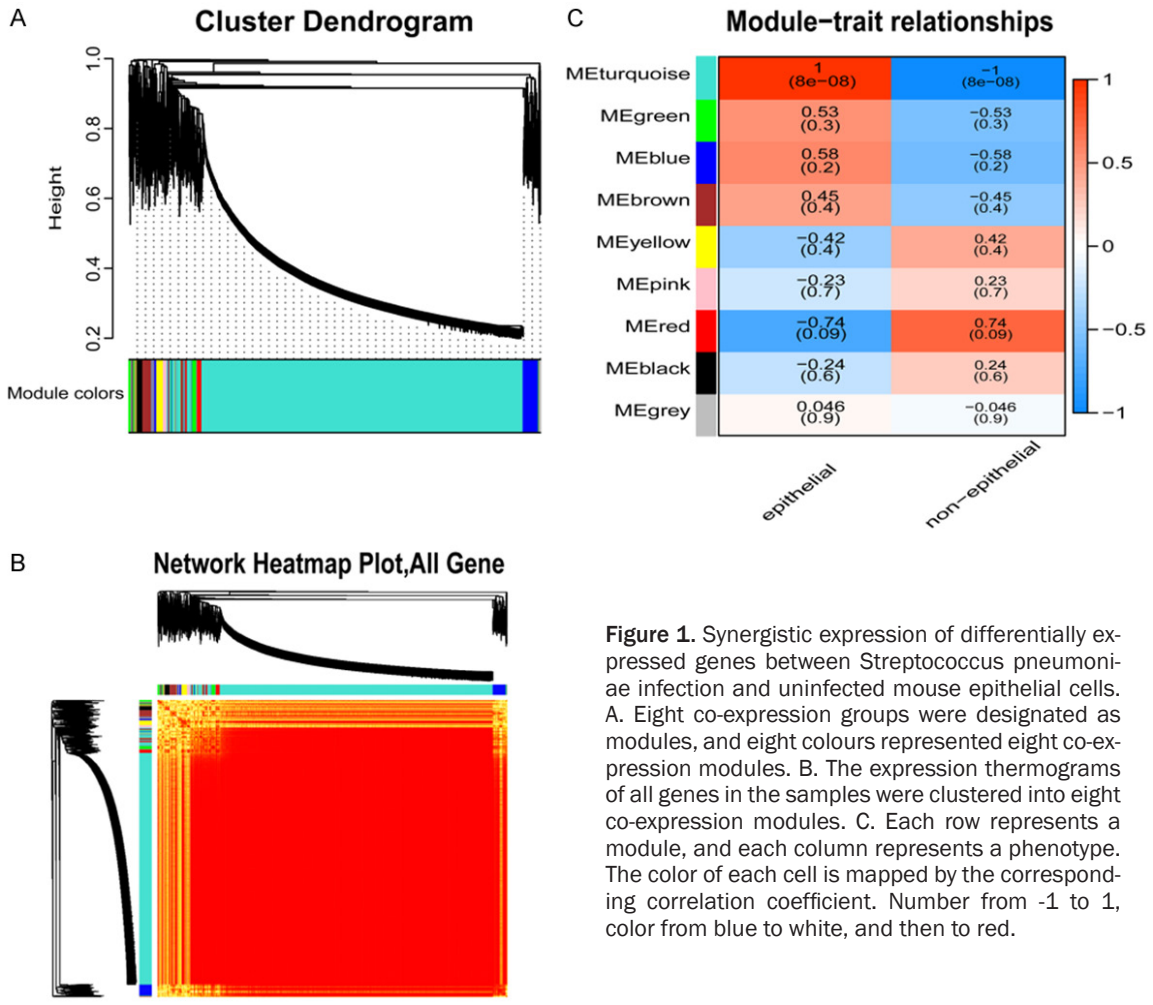

Figure 1. Synergistic expression of differentially expressed genes between Streptococcus pneumoniae infection and uninfected mouse epithelial cells. A. Eight co-expression groups were designated as modules, and eight colours represented eight co-expression modules. B. The expression thermograms of all genes in the samples were clustered into eight co-expression modules. C. Each row represents a module, and each column represents a phenotype. The color of each cell is mapped by the corresponding correlation coefficient. Number from -1 to 1, color from blue to white, and then to red.

mice are mainly related to purine metabolic signaling pathway (**Figure 2B**). For the most regulated functions and pathways of genes in dysfunction module, we can think that genes play the most decisive role in dysfunction module, driving dysfunction module.

TF and ncRNA driving the development of pneumonia

We conducted pivot analysis of co-expression module genes based on the targeting regulation of TF and ncRNA to explore the key transcriptional regulators regulating the development of pneumonia. Results (**Figure 3A**, **3B**) showed that 177 ncRNAs involved 194 ncRNA-module regulatory pairs and 22 transcription factors involved 44 TF-module target pairs. In addition, the number of pivot regulatory modules was statistically analyzed. In ncRNA pivot analysis, three functional dysfunction modules were participated by microRNA 181a-5p. In TF pivot analysis, transcription factors such as Apc, Batf2 and Cebpg, which regulate more module genes, were identified. These transcription factors and ncRNA may regulate the development of pneumonia by mediating dysfunctional modules. Therefore, we identified these potential regulators as dysfunctional molecules in the development of pneumonia.

Discussion

Studies have demonstrated that the incidence of pneumonia in children under 5 years old is the highest [1]. Streptococcus pneumonia is the main cause of pneumonia. To prevent and treat pneumonia, it is necessary in order to study its pathogenesis. However, the effect of current studies on the host molecular process leading to the occurrence and development of pneumonia is not entirely clear. With the furth-

Figure 2. Modular gene participates in function and pathway identification of pancreatic cancer dysfunction module (excerpts). A. GO function of module gene. The darker the color, the stronger the significance of enrichment. The larger the circle, the larger the proportion of module genes in GO functional entry genes. B. KEGG signaling pathway of modular genes. The darker the color, the stronger the significance of enrichment. The larger the circle, the larger the proportion of module genes to KEGG pathway entry genes.

er research of countless scholars, it is believed that bacterial envelope compounds trigger immune and inflammatory responses through chemokines/cytokines [18]. As epithelial cells are the first line of defense for most microorganisms, up-regulation of chemokine expression can promote biofilm formation, which is an important innate defense response of host to invasive microorganisms such as Streptococcus pneumonia [19, 20]. So the first step for Streptococcus pneumoniae to guide the development of pneumonia is through epithelial cells. The data of this study and other scholars also show that there are differences in gene expression between pathogenic and non-viral Streptococcus pneumonia during the interaction with human lung epithelial cells [21]. It is shown that the interaction between Streptococcus pneumoniae and host mucosal epithelial cells is a prerequisite for the occurrence of Streptococcus pneumoniae disease [22]. The results of this difference show that Streptococcus pneumoniae infection in the lung is related to epithelial cells. The infection process involves microbial cell surface receptors, which interact with the host extracellular matrix components to promote bacterial colonization and transmission [23]. Regarding the interaction between Streptococcus pneumoniae and pulmonary epithelial cells, data indicate that Streptococcus pneumoniae promotes uptake by reticulin-mediated endocytosis (CME) and fossa-mediated endocytosis to enter cells [24, 25]. Further analysis of membrane proteins revealed that Streptococcus pneumoniae surface protein C (PspC) is the main adhesion protein of Streptococcus pneumoniae. It interacts with the outer region of human polymeric immunoglobulin receptor (pIgR) produced by epithelial cells in a definite way [26]. Meaningful differences in this study also indicate the potential role of infected pulmonary epithelial cells. PfbB significantly improves the adhesion ability of Streptococcus pneumoniae to human epithelial cells by directly interacting with fibronectin [27]. It can be seen that Streptococcus pneumoniae is a specific combination of epithelial cells leading to pneumonia, and the mechanism of pneumonia caused by Streptococcus pneumoniae, is somehow caused by cell necrosis. Regardless of the fact that necrosis is a major pathway of cell death, experimental data show that necrosis can occur without death receptor signal (the main adhesion protein of Streptococcus pneumoniae) [28]. Therefore, the specific gene expression of Streptococcus pneumoniae and epithelial cells led to the occurrence and development of pneumonia, but not necessarily lead to cell necrosis. In this study, data of GSE71623 in GEO database were collected and analyzed. The pathogenic genes of GSE71623 were further analyzed. Eight functional disorder modules were obtained. Key genes of each module were identified based on purposeful disorder module, and core genes such as Aqp5 and Mak6 were obtained. The data showed that Pepo enhanced the production of

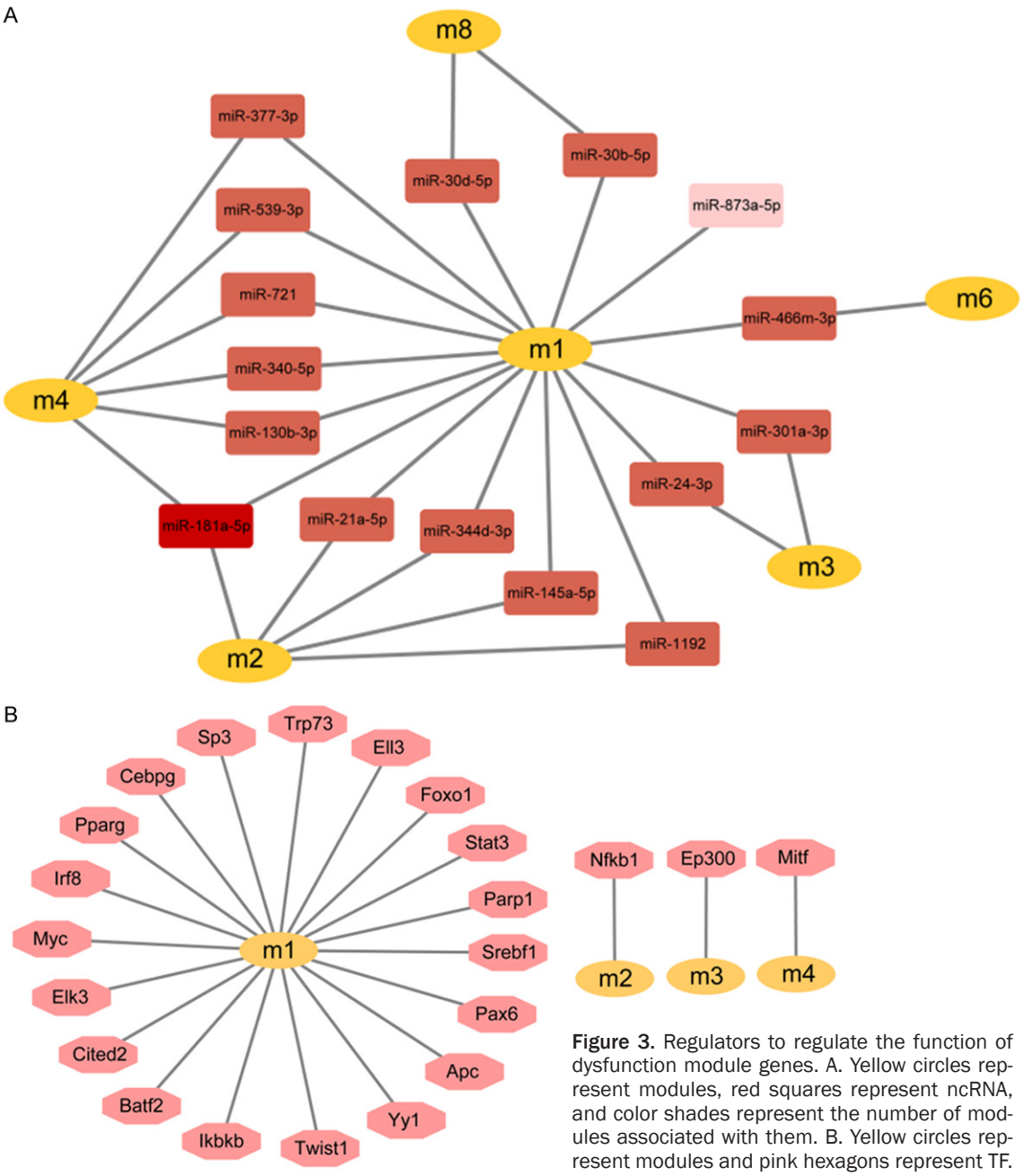

Figure 3. Regulators to regulate the function of dysfunction module genes. A. Yellow circles represent modules, red squares represent ncRNA, and color shades represent the number of modules associated with them. B. Yellow circles represent modules and pink hexagons represent TF.

IL-8 and IP-10 in BEAS-2B in a MAPKs-PI3K/Akt-p65 dependent manner, which may play a critical role in the pathogenesis of pneumonia [29]. The expression of Nod1 and Nod2 was upregulated in mouse lung tissue and epithelial cell BEAS-2B after Streptococcus pneumoniae infection [30]. This study and other comparative studies have shown that the genome damage of host cells induced by Streptococcus pneumonia aggravates its toxicity and pathogenesis, making DNA repair a potentially important susceptibility factor for pneumonia patients [31]. However, unlike epithelial cells, the effect of Streptococcus pneumoniae on IAV replication may be different between in vivo co-infection of human upper respiratory tract and in vitro co-infection of primary polarization of human bronchial epithelial cells due to the influence of host secretory factors [32].

In this study, after the functional impairment module and core genes were obtained, the functions of the genes of interest were discussed. In the analysis of the pathways invol-

ved in genes of interest, the mmu00230 pathway involved in eight modules was obtained. Its function is linked to purine metabolism. It is suggested that the epithelial specificity of lung gene expression in Streptococcus pneumoniae infection may affect the occurrence and progress of pneumonia by participating in or altering the metabolic pathway of purine. Lipoteichoic acid (LTA) is a key pro-inflammatory component in the cell wall of Gram-positive bacteria. R-roscovitine is a purine analogue and a potent inhibitor of cyclin-dependent kinase (CDK)-1, -2, -5 and -7. It has the capacity to inhibit cell cycle and induce apoptosis of polymorphonuclear cells (PMN) [33]. At the same time, the molecular functions of interesting genes were analyzed. There were 8 modules involved in 27 molecular functions, most of which were linked to the activity of enzymes, including S-adenosylmethionine-dependent methyltransferase activity, ATPase activity and so on. Studies have shown that S-carboxymethyl cysteine (S-CMC) regulates the adhesion of Streptococcus pneumoniae to epithelial cells by acting on cells and bacteria, so S-CMC may be an inhibitor of various epithelial receptors interacting with Streptococcus pneumoniae [34, 35]. Among them, the molecular function of ATPase activity also exists in eight functional modules. Studies have shown that during Streptococcus pneumoniae pneumonia, the epithelium is susceptible to a large amount of hydrogen peroxide as a product of oxidative metabolism of host and pathogen [36]. Therefore, it can be seen that the potential effect of epithelial cells on pneumonia may be related to the influence of ATPase activity.

The results showed that there were 177 ncRNAs and 22 transcription factors that had significant regulatory effects on module genes. In addition, the number of pivot regulatory modules was analyzed by statistical analysis. In ncRNA and TF pivot analysis, potential regulatory factors such as microRNA-181a-5p, Apc, Batf2, Cebpg, Cited2, Elk3, Ell3, Ep300, Foxo1, Ikbkb, Irf8 were identified as dysfunctional molecules in the pathogenesis of pneumonia. After the above analysis, regarding the prevention and treatment of pneumonia, we can start from the hypothetical role of epithelial cells in pneumonia, from the following points for prevention and treatment. First, maintaining the integrity of the pulmonary epithelial barrier is essential to ensure breathing and proper oxygenation in patients with various pulmonary inflammation [37]. Plasminogen binding to Streptococcus pneumoniae reduced the adhesion to epithelial cells [38]. Therefore, the study of the potential role of epithelial cells can enable clinical prevention of pneumonia from the first line of defense. Pore-forming toxin pyrolysis (PLY) is the main virulence factor [39]. Therefore, CBPA of Streptococcus pneumoniae can induce the transcription and release of pro-inflammatory molecule [40] in human alveolar epithelial cells. For the treatment and prevention of pneumonia, many studies have shown that LGG, NE and PcpA antibodies significantly inhibit the adhesion of Streptococcus pneumoniae to epithelial cells [41-43], while classical DCs, PhtD, PcpA and thickness proteins are considered to have potential as alternatives to conjugated vaccines [44, 45]. From the study of epithelial specificity of gene expression in lung infected by Streptococcus pneumoniae, it can be seen that the epithelial cells may guide the occurrence and development of pneumonia by regulating the purine metabolic pathway and ATPase activity. This study cannot only make us more aware of the potential role of epithelial cells in pneumonia, but also provide a reference for clinical treatment.

Disclosure of conflict of interest

None.

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