Bioinformatics analysis of potential key genes and pathways in neonatal necrotizing enterocolitis

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Research Article

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Abstract

Objective

To detect differentially expressed genes in patients with neonatal necrotizing enterocolitis (NEC) by bioinformatics methods and to provide new ideas and research directions for the prevention, early diagnosis and treatment of NEC.

Methods

Gene chip data were downloaded from the Gene Expression Omnibus database. The genes that were differentially expressed in NEC compared with normal intestinal tissues were screened with GEO2R. The functions, pathway enrichment and protein interactions of these genes were analyzed with DAVID and STRING. Then, the core network genes and significant protein interaction modules were detected using Cytoscape software.

Results

Overall, a total of 236 differentially expressed genes were detected, including 225 upregulated genes and 11 downregulated genes, and GO and KEGG enrichment analyses were performed. The results indicated that the upregulated differentially expressed genes were related to the dimerization activity of proteins, while the downregulated differentially expressed genes were related to the activity of cholesterol transporters. KEGG enrichment analysis revealed that the differentially expressed genes were significantly concentrated in metabolism and fat digestion and absorption pathways. Through STRING analysis, 9 key genes in the protein network interaction map were identified: EPCAM, CDH1, CFTR, IL-6, APOB, APOC3, APOA4, SLC2A and NR1H4.

Conclusion

Metabolic pathways and biological processes may play important roles in the development of NEC. The screening of possible core targets by bioinformatics is helpful in clarifying the pathogenesis of NEC at the gene level and in providing references for further research.

Introduction

Neonatal necrotizing enterocolitis (NEC) is caused by ischemia and hypoxia of the intestinal wall and results from a variety of factors that damage the blood supply of the intestinal mucosa. NEC mainly impacts preterm infants, especially those with very low birth weight. The incidence rate ranges from 7% to 11%[1] and is associated with high mortality. It has been reported that the mortality of
NEC children with low birth weight was 41.7%, while that of children with very low birth weight was 50.2% [2]. Furthermore, the mortality of children with extensive intestinal necrosis is nearly 100%, which is a significant cause of neonatal death [3]. After years of efforts, the mortality rate remains at 20% to 30% and is even higher in cases requiring surgical treatment [4]. NEC can damage children's intellectual development and is an important risk factor for long-term intellectual disability [5,6]. With regard to the diagnosis and treatment, it is often difficult to accurately predict intestinal necrosis in the early stage of the disease by using a single indicator, while the combined application of multiple indicators helps more [7,8]. Bell et al. formulated the NEC diagnostic standard in 1978 [9], then Walsh and kliegman revised it in 1987 [10]. But in view of the fact that NEC has many manifestations in recent years [11], both of the "two out of three" standard for premature infants developed by the International Association of Newborns and the Vermont Oxford Network diagnostic standard based on Bell staging have included abdominal B-ultrasound examination [12]. However, these are far from enough for the early diagnosis of NEC in clinical practice. The pathogenesis of NEC has been documented closely relating to genetic factors [13]. Recent development in molecular analysis techniques have allowed more advanced genetic analysis methods available in laboratorial or even clinical circumstances. This makes more comprehensive and accurate diagnosis of NEC possible at its earlier stages. However, to our best knowledge, literature on this topic is still few.

To establish a hybrid gene analysis method to early reveal NEC, this study, based on the Gene Expression Omnibus (GEO) and used the GSE46619 dataset, is aimed to identify the differentially expressed genes (DEGs) in children with NEC by bioinformatics methods. In addition, functional (GO), signal pathway (KEGG) and protein-protein interaction (PPI) network integration analyses of differentially expressed genes were also performed, and meaningful PPI modules and key genes were detected. This study provides a reference for further research on the molecular mechanism of NEC.

Materials And Methods

Data sources

The GeneChip dataset GSE46619 was downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/), which was submitted by NGPC, CHAN KY, LEUNG KT, etc., and included 9 samples from patients, 5 of whom underwent surgery on intestinal tissues and 4 control cases consisting of patients with other noninflammatory congenital intestinal diseases who underwent other surgeries.

DEG screening

The GSE46619 dataset was analyzed with the online network analysis tool GEOR2 in the GEO database. DEGs with adjusted P < 0.05 and $|\log_2 FC| > 2$ were selected.

Bioinformatics analysis of DEGs
GO enrichment and KEGG pathway analyses were performed on the related DEGs in NEC using the online analysis software DAVID (https://david.ncifcrf.gov/). The differences were statistically significant when P < 0.05.

Construction and analysis of the PPI network

PPI network was used to help the analysis of gene or protein interactions, while string online database (http://string db.org/) was used to help the construction of PPI network diagram. In order to reveal the potential interaction between differentially expressed genes, the PPI network diagram data constructed by string was imported into a Cytoscape software, while the mcode plug-in was exerted to analyze the sub network structure of PPI. Degree of connective is a parameter to evaluate the connection tightness in network. The higher the connectivity, the closer the connection and the stronger the interaction with other nodes in the whole network, thus enhancing the stability of the whole network. Those genes with higher connectivity will be regarded as the key ones in the differentially expressed genes. The three most significant modules and key genes were screened out using the MCODE plug-in of Cytoscape.

Results

DEG screening

Based on P < 0.05 and |log2FC| > 2, 236 differentially expressed genes were screened, including 225 upregulated genes and 11 downregulated genes. The top 10 differentially expressed genes are shown in Table 1.

GO enrichment and KEGG analyses of DEGs

GO enrichment and KEGG analyses were performed on 236 DEGs with the DAVID online tool, and the results are displayed in Table 2. (1) Biological process: Differentially expressed genes were significantly concentrated in the processes of heterologous metabolism, drug response, redox process, inflammatory reaction, and carbohydrate metabolism, among others. (2) Cell component: Differentially expressed genes were dramatically accumulated in the components of membrane, exosomes, plasma membrane, components of plasma membrane, and sarcoplasmic membrane, among others. (3) Molecular function: Differentially expressed genes were significantly concentrated in protein homodimeric activity, transporting activity, actin filament binding, iron ion binding, actin binding, and monooxygenase activity, among others. KEGG enrichment analysis demonstrated that the differentially expressed genes were significantly enriched in metabolism, fat digestion and absorption, protein digestion and absorption, chemical carcinogenesis, carbohydrate digestion and absorption, and retinol metabolism pathways (Fig.1).

PPI analysis

All differentially expressed genes were upload to string database and obtain PPI network data. The PPI network data was imported into a Cytoscape software for further analysis of the potential biological
information of PPI network diagram, and the key factors of the nine hub nodes with the highest value > 15 were selected: epithelial cell adhesion molecule (EPCAM), cadherin 1 (CDH1), cystic fibrosis transmembrane conduction regulator (CFTR), interleukin-6 (IL-6), APOB, APOC$_3$, apolipoprotein A- (APOA$_4$), solute carrier family 2-facilitated glucose transporter member 1 (SLC2A), and nuclear receptor subfamily 1, group H, member 4 (NR1H$_4$). Two genes were downregulated, and seven genes were upregulated. A PPI network was constructed (Fig. 2) with 204 nodal proteins and 528 edges, and p < 0.001. The PPI network diagram was analyzed using the MCODE plug-in of Cytoscape, and the three most significant modules were selected (Fig. 3).

**Discussion**

**Enrichment analysis of NEC**

In this study, bioinformatics analysis was performed on the gene expression profiles of intestinal tissues of 5 NEC patients and 4 non-NEC patients, and the differentially expressed genes involved in the occurrence and development of NEC were identified. Compared with those of the control group, 225 differentially expressed genes were upregulated and 11 were downregulated in NEC patients. The results of the GO analysis indicated the following expression patterns: (1) Biological process: The differentially expressed genes were significantly concentrated in the processes of heterologous metabolism, drug response, redox process, inflammatory reaction, and carbohydrate metabolism; (2) Cell component: The differentially expressed genes were dramatically clustered in the components of membrane, exosomes, plasma membrane, components of plasma membrane, and sarcoplasmic membrane; (3) Molecular function: Differentially expressed genes were concentrated in protein homodimeric activity, transport activity, actin filament binding, iron ion binding, actin binding and monooxygenase activity. Albeit variable factors affecting the blood supply of the intestinal mucosa, ischemia-induced necrosis is still regarded as the core pathological feature in the development of NEC. In the diagnosis of NEC, nonspecific biomarkers, such as acute C-reactive protein (CRP), procalcitonin (PCT), serum amyloid A, platelet-activating factor, tumor necrosis factor-α, interleukin-6, interleukin-8 and other nonspecific biomarkers, are mediators of pro- and anti-inflammatory pathways of the immune system and play important roles in the pathogenesis of NEC. GO analysis revealed that IL-6 can be measured to distinguish NEC from non-septicemia-related diseases, although it is difficult to distinguish NEC from septicemia. Chatziioannou et al. compared the omics data of children with NEC and children with septicemia by LC-MS/MS mass spectrometry and identified the protein (APOA4) that could better distinguish the two diseases\[^{14}\]. The results are consistent with those in this study, which suggest that APOA4 may be a gene closely related to NEC that can be employed in distinguishing NEC from some other diseases.

KEGG enrichment analysis revealed that the differentially expressed genes were significantly concentrated in metabolism, fat digestion and absorption, protein digestion and absorption, chemical carcinogenesis, carbohydrate digestion and absorption, and retinol metabolism pathways, among others. Currently, Toll-like receptor 4 (TLR4) is the most frequently studied signaling pathway in NEC. TLR4,
which is highly expressed in intestinal epithelial cells of preterm mice and humans, is essential for NEC development\[15,16\]. Inhibitors of TLR4 signaling include small molecules, amniotic fluid, breast milk and intestinal epithelium; the absence of TLR4 can alleviate the severity of NEC\[17,18\]. TLR4, which plays a key role in the pathogenesis of NEC, is widely expressed in intestinal epithelial cells and intestinal lymphocytes\[19\]. After TLR4 is activated by the corresponding pathogenic microorganism, it initiates the innate immune response and further the downstream NF-kB signaling pathway and mediates the expression and release of the inflammatory factors such as IL-1, IL-6, IL-8 and TNF-\(\alpha\). Studies have shown that the expression of TLR4 in gut epithelium is increased in intestinal inflammation of human and mouse model, and overexpression of TLR4 leads to a signaling cascade that initiates nuclear translocation of NF-kB and promotes overtranscription of proinflammatory cytokines, inducing the incidence of NEC\[20\]. IL-6 binds with important molecules of the innate immune system to activate TLR4, which stimulates intracellular signaling and produces inflammatory cytokines. Myeloid differentiation-2 (MD-2) and GM2 activator (GM2A) proteins are members of the MD-2-related lipid recognition (ML) family. MD-2 is a very important component of the intestinal TLR4 innate immune signaling pathway\[21\]. MD-2 has been proven to bind with TLR4 to form a heterodimer, thus forming a complete binding site for lipopolysaccharide (LPS)\[22\], and hence, MD-2 is a necessary accessory molecule for TLR4 to bind with LPS\[23\]. Cells expressing TLR4 alone or TLR4 and mutant MD-2 showed low LPS reactivity\[24\]. MD-2 is an important component of the CD14-TLR4/MD-2 receptor complex, which can be used to identify the components of the bacterial cell wall\[25\]. Therefore, genetic polymorphisms of the MD-2 gene promoter or its exons can significantly affect the transcriptional activity of the MD-2 gene or LPS-induced signal transduction\[26\], resulting in abnormal immune responses. These findings suggested that such changes in biological processes and signaling pathways might play important roles in the evolution of NEC.

PPI network analysis led to the identification of key genes, the most important of which are EPCAM, CDH1, CFTR, IL-6, APOB, APOC3, APOA4, SLC2A and NR1H4. Interleukin 6 (IL6) is one of the genes encoding the cytokine interleukin family that play a role in inflammation and B cell maturation. In addition, it has been proved that the protein encoded by this gene mainly induces inflammatory response by binding to interleukin-6 receptor (IL6R). It is produced at the site of acute and chronic inflammation where it is released into serum\[27\]. IL6 regulates the differentiation of various cells of the immune system including macrophages, T cells and several other cells\[28,29\]. In cells such as monocyte, macrophage, fibroblast, and endothelial cell, IL6 expression is regulated by inflammatory pathways, for example, NF-KB, or AP-1 (Activator protein1). The studies by Gross et al. demonstrated that serum concentrations of IL6 in patients with inflammatory bowel disease, as compared to healthy controls, increased significantly\[30\]. Further study found that high expression of IL6 was common in patients with Crohn's disease and ulcerative colitis and closely associated with disease activities\[31\]. Louis et al. have used high IL6 serum levels as the biomarkers to predict for stopping recurrence in patients with Crohn's disease\[32,33\]. In fact, IL6 serum levels show a higher disease activity association than the more extensively used biomarker C-reactive protein level\[34\]. It has been proved that IL6 expression in inflammatory bowel disease may derived from the activation of a variety of cells including monocyte,
intestinal epithelial cell and lamina propria monocyte\cite{35}. IL6 play a key role in the differentiation of Th17 cells from natural CD4+T cell precursor and the transformation of Treg cells into IL17+Treg cells. Ma Fei and other authors found that Treg cells producing CCR9+IL17 in peripheral blood were significantly increased among children as well as mice with NEC. IL6 can promote the transformation of CCR9+Treg cells back to CCR9+IL17+Treg cells. When IL6 signal is blocked, transduction can inhibit the transformation\cite{36}. CDH1, a gene encoding the classical cadherin of the cadherin family and the protein that maintains intercellular tight connection of intestinal tract. Current researches focused mainly on its mutations and actions on cancer proliferation, invasion and metastasis. CDH1 has also been identified as a susceptibility gene for inflammatory bowel disease, which may increase the risk of Crohn's disease and ulcerative colitis\cite{37,38}. A recent study in Netherlands conducted genetic testing on 821 patients with ulcerative colitis and 1260 healthy individuals, and determined that rs1728785 of CDH1 was mutated, resulting in an increased risk of ulcerative colitis by 1.23 times\cite{39}. Similar results have been demonstrated by a study from the University of Toronto in Canada\cite{40}. In this current research we think that the effect of CDH1 mutation in NEC is mainly on intestinal tight junction. The mutation induced distintegration of intestinal mucosal barrier. Apolipoprotein B (APOB), a gene encodes the product of apolipoprotein in chylomicrons and low-density lipoprotein (LDL). It has two plasmaic subtypes of apoB-48 and apoB-100. ApoB is encoded by a single gene of a single long-chain mRNA. Recent researches are mainly limited to diseases such as apoB gene or its regulatory region mutation related hypolipidemia, and few on intestinal diseases.

Limitations

Not like that by using RNA seq-technique that sequences the whole transcriptome, the dataset used in this article only give a profile of the predefined transcripts or genes through hybridization, so technically it did not provide a full picture of gene expression. Also, since the dataset is not single-cell-based array, cell specific gene profile is impossible to be concluded (Immune cells, Endothelial cells, Epithelial cells). Besides, the size of nine samples in this study is very small, resulting in a weakened evidence out of them.

Conclusions

In summary, this study used bioinformatics methods to apply NEC gene chip data to the GEO database, screened the differentially expressed genes, and performed GO and KEGG enrichment analyses to identify the genes and signaling pathways that might be related to NEC. IL-6 and TLR4 may play important roles in the incidence and development of NEC. In addition, the roles of eight key genes in NEC, in addition to IL-6, are not clear, and further study is encouraged.

Declarations

Statement of financial support: This work was supported by the Chongqing Science and Technology Commission (2020MSXM028).
Disclosure statement: The authors declare no conflicts of interest.

Authors’ Contributions:

All six authors made substantial contributions to the study and manuscript and met the criteria for authorship defined in the author instructions:

Xuexiu Liu contributed to acquisition, analysis and interpretation of the data and acquisition, analysis and interpretation of the data and the drafting and final approval of the manuscript.

Xianhong Zhang, Luquan Li, Jianhui Wang, Yanhan Chen provided technical support and conceptual advice.

Liping Wu designed the study.

All authors read and approved the final manuscript.

The GEO data belong to public databases. The patient data in the database were ethically obtained with the appropriate approvals. Users can download relevant data for free for research purposes and for publishing relevant articles. Our study is based on open source data; thus, there are no ethical issues or other conflicts of interest.

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Fig1. Schematic Diagram of KEGG Pathway (a, b)

Fig2. Schematic Diagram of PPI Network

Fig3. Schematic Diagram of Modular( a,b,c)

Table 1. Upregulated DEGs (Top 10)

Table 2. KEGG analysis of DEGs

Table 3. GO analysis of DEGs

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Tables

Table 1. Up regulation of DEG(TOP10)

| Serial number | P value       | Gene name | Gene annotation               |
|---------------|---------------|-----------|-------------------------------|
| 1             | 0.0000713     | CLRN3     | clarin3                       |
| 2             | 0.00007123    | MTTP      | microsomal triglyceride transfer protein |
| 3             | 0.0000711     | ALDOB     | aldolase, fructose-bisphosphate B |
| 4             | 0.00007093    | ANXA13    | annexin A13                   |
| 5             | 0.00007093    | OTC       | ornithine carbamoyltransferase |
| 6             | 0.00007093    | APOA4     | apolipoprotein A4             |
| 7             | 0.00006994    | FABP2     | fatty acid binding protein 2  |
| 8             | 0.00006994    | PRSS8     | protease, serine 8            |
| 9             | 0.00006958    | AADAC     | arylacetamide deacetylase     |
| 10            | 0.0000623     | VIL1      | Villin 1                      |

Table 2. KEGG analysis of DEG

Table 3. GO analysis of DEG
| pathway          | description                               | Gene number | P         | Gene                                                   |
|------------------|-------------------------------------------|-------------|-----------|--------------------------------------------------------|
| hsa04975         | Fat digestion and absorption              | 9           | 2.61e-07  | ABCG8. FABP2. ABCG5. DGAT1. MOGAT3. MOGAT2. MTTP. APOA4. APOB |
| hsa04976         | Bile secretion                            | 9           | 1.19e-0.5 | ABCG8. S4C9A3. ABCG5. ABCB1. NRIH4. ATPIA1. S4C51B. CFTR. SULT2A1 |
| hsa01100         | Metabolic pathways                        | 35          | 1.19e-0.5 | SDS1. ST6GALNAC1. DDC. TPH1. ACY1. MOGAT3. GDA. GBA3. CYP2C19. FUT2. PTGS2 |
| hsa04974         | Protein digestion and absorption          | 8           | 0.00039   | S6C9A3. ACE2. S4CI5A1. S4C6A19. MEPIA. KCVJ13. S4CIA1. ATPIA1 |
| Hsa04973         | Carbohydrate digestion and absorption     | 6           | 0.00039   | HKDC1. S1. S4C2A2. ATPIA1. 4CT. SLIC37A4               |
| Type                | Classification number | Gene number | P value      | Gene function                                           |
|---------------------|-----------------------|-------------|--------------|--------------------------------------------------------|
| GOTERM - BP         | GO: 0007586           | 13          | 8.25e-07     | Digestion                                              |
|                     | GO: 0015711           | 22          | 1.58e-06     | Organic anion transport                                |
|                     | GO: 0006629           | 38          | 1.58e-06     | Lipid metabolism process                              |
|                     | GO: 0044281           | 47          | 2.19e-06     | Metabolic process of small molecules                  |
|                     | GO: 0006820           | 24          | 2.19e-06     | Anion transport                                        |
| GOTERM – BMF        | GO: 0008509           | 17          | 0.00011      | Activity of anion transmembrane transporters           |
|                     | GO: 0005215           | 33          | 0.00021      | Transport activities                                   |
|                     | GO: 0022804           | 16          | 0.00034      | Active transmembrane transporter activity              |
|                     | GO: 0022857           | 29          | 0.00035      | Transmembrane transporter activity                     |
|                     | GO: 0051015           | 10          | 0.0013       | Actin filament binding                                 |
| GOTERM - CC         | GO: 0045177           | 32          | 7.61e-17     | The apical part of a cell                              |
|                     | GO: 0016324           | 29          | 2.38e-10     | Parietal plasma membrane                               |
|                     | GO: 0005903           | 15          | 7.50e-11     | Brush border                                           |
|                     | GO: 0098590           | 40          | 2.22e-10     | Plasma membrane area                                   |
|                     | GO: 0044425           | 113         | 6.96e-09     | Membranous part                                        |

**Figures**
Figure 1

See image above for figure legend
Fig2. Schematic Diagram of PPI Network. The PPI network was constructed with 204 nodal proteins and 528 edges. The proteins were represented by the nodes, and the predicted functional associations were represented by the edges.

**Figure 2**

See image above for figure legend
Figure 3

See image above for figure legend