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Identification of candidate genes for necrotizing enterocolitis based on microarray data

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Abstract.

Necrotizing enterocolitis (NEC) is one of the most serious diseases that could threaten the life of neonates. However the current opinions about the pathogenesis or how to prevent or treat the disease are still ambiguous. The purpose of the present study was to identify the key genes of this disease and provide new insights into the mechanism of NEC. The gene expression data of GSE46619, including 5 specimens from NEC patients and 4 samples from surgical-control infants, were collected from Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) were screened with regard to NEC versus surgical-control group using Limma package in R software and Gene Ontology (GO) enrichment analysis and pathway enrichment analysis were conducted by means of Database for Annotation, Visualization and Integrated Discovery (DAVID) website subsequently. Furthermore the protein-protein interaction (PPI) network for DEGs was constructed using Cytoscape software and the most highly connected module was extracted using MCODE plugin from the PPI network. Moreover，the significantly enriched sub-pathways were identified using iSubpathwayMiner package in R software. A total of 2629 DEGs were screened out between NEC and control samples, including 367 up-regulated genes and 2262 down-regulated genes and they involved in different GO terms and pathways which may be associated with NEC onset and progression. PPI network and module analysis revealed that several genes were defined as hub genes including AGT, IL8 and KNG1. The sub-pathway analysis screened out 189 significantly enriched sub-pathways, including Tryptophan metabolism, Fatty acid metabolism, and Arachidonic acid metabolism. Genes in the corresponding sub-pathway, such as ACACB and CAT were regarded as critical genes in NEC. QRT-PCR was also conducted to identify the expression of the five key genes (AGT, IL8, KNG1, ACACB and CAT) in NEC samples. These findings have identified several hub genes (e.g., AGT, IL8, KNG1, ACACB and CAT) which were presumed

to serve critical roles in NEC.

Key words: necrotizing enterocolitis；differentially‐expressed genes；protein‐protein interaction network；module analysis；sub‐pathway analysis

1. Introduction

Necrotizing enterocolitis (NEC) is a life-threatening disease which is characterized by intestinal necrosis and mainly oculars in premature neonates(<1500g or <32weeks) (Thompson and Bizzarro, 2008). The overall incidence rate is approximately 1 in 1000 live births while the proportion of NEC in very low birth weight infants is up to 7%, with an estimated 15 to 30% mortality risk(Llanos et al., 2002; Henry and Moss, 2009). The pathophysiology of NEC has been tried to be identified among various studies. The multifactorial thought to be involved in the pathogenesis of NEC are: enteral feeds of intestinal immaturity, the intraluminal microbiome, inflammation and local ischemia and/or reperfusion injury (Somaschini et al., 2012; Neu, 2014; Elgin et al., 2016). However, the exact mechanism underlying NEC is yet to be fully elucidated.

In 2014, Chan et al used a whole‐genome microarray approach to analyze genes that differed between NEC and surgical-control, and acquired gene profiles that could be used for downstream analysis(Chan et al., 2014). Based on the obtained data, the present study aimed to provide an insight into the molecular mechanism of the development and progression of NEC by Gene Ontology (GO) and pathway enrichment analyses. Besides, interaction associations between these DEGs were screened out using the protein‐protein interaction (PPI) network and several key genes were selected, which may be potential for clinical treatment and diagnosis of NEC in the future. In addition, network module analysis was subsequently performed according to the PPI network. Besides, the significantly enriched sub-pathways were identified and genes in the corresponding sub‐pathway might contribute to the pathogenesis of NEC based on the molecular level.

Previous studies only focused on the DEGs and selected the most significant genes for further study. In the recent years, the topological analyses have been applied to molecular networks including protein interaction networks, whose nodes are proteins linked to each other via physical interactions. In this study, we identified several key genes derived from the common network representing both PPI and risk sub-pathway information. The differentially expressed genes (DEGs) were screened based on the gene expression microarray of NEC in GEO

database and several critical genes were identified via the construction and analysis of PPI networks. Our results may provide a new insight into the understanding of NEC pathogenesis and the identified hub genes may serve as potential targets for treatment of NEC.

2. Materials and methods

2.1. Affymetrix microarray data.

The gene expression data of GSE46619, based on the GPL6244 [HuGene-1_0-st] were collected from Gene Expression Omnibus (GEO) database, which was deposited by Chan KY et. A total of 9 specimens,including 4 surgical-control samples and 5 NEC specimens were used for subsequent analysis.

2.2. Data preprocessing and DEGs screening.

The procedure of probe-level data in CEL files was conducted by affy package in R (Chan et al., 2014). Background correction was carried out using the robust multi-array averagy (RMA) method, followed by quantile normalization and probe summarization (Irizarry et al., 2003; Smyth, 2004). The limma package in R was applied to identify DEGs between NEC and surgical-control samples. In this analysis, p value < 0. 05 and log2 (fc)|>1 were used as the cutoff criteria.

2.3. Functional enrichment analysis of the DEGs.

To investigate the main functional pathways of NEC, Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to perform the Gene Ontology (GO) analysis and KEGG pathways enrichment analysis of DEGs (Dennis et al., 2003). Criteria for this step were set as p value<0. 05 and DEG count ≥2.

2.4. Construction and analysis of Protein-protein Interaction (PPI) network of DEGs.

Based on the DEGs obtained above, we extracted their interacting partners from Search Tool for the Retrieval of Interacting Genes (STRING; version 10, http: //string-db. org) (Szklarczyk et al., 2015). The combined score > 0. 4 was the PPI value. Cytoscape 3. 4. 0 was used for visualization and then to calculate the properties of the PPI network under default parameters. Only connected nodes were retained for further analysis. After obtaining the PPI network, the Network Analyzer plug-in of Cytoscape was utilized to analyze the topology property of the networks(Shannon et al., 2003). Next, connectivity degree analysis was performed and key nodes (i. e., hub genes) were identified. To explore more specific regulatory relationship of proteins, we conducted the module analysis of

the network by using the MCODE1. 4. 1 (a plug for Cytoscape) with default setting (Bandettini et al., 2012).

2.5. Risk sub-pathway analysis.

To identify the significantly enriched sub-pathways based on DEGs among the PPI network, iSubpathwayMiner was adopted for graph‐based construction and analysis of KEGG pathways supported by the R platform(Li et al., 2009). The k-clique method is used to define metabolic sub-pathways based on gene sets. Here, the value of k was set 3 which mean that the distance among all nodes in one sub-pathway is not greater than 3. After importing the DEGs into SubpathwayMiner, genes which had higher degrees in the PPI network enriched in the sub‐pathways were identified as the critical genes in NEC.

2.6. Samples collection and ethics statement

In this study, we collected 32 NEC small bowel specimens from patients at Children's Hospital of Nanjing Medical University between 2011 and 2018. All selected patients were confirmed by histology. Thirty-two corresponding normal small intestine tissues without inflammatory conditions were also collected. Immediately following removal, all tissues were stored at -80℃ before using. Each patient enrolled in the study has signed informed consent and this whole study was authorized by the Institutional Ethics Committee of Nanjing Medical University. Patients' clinical features are summarized in Table 4.

2.7. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from tissues and cells were extracted using TRIzol reagent (Life Technologies, USA). For mRNA detection, each RNA sample was reverse transcribed into cDNAs using the reverse transcription kit (Takara, Tokyo, Japan). The qRT-PCR was employed to measure the levels of mRNAs using the comparative Ct method. GAPDH was considered as the normalization control for mRNA. All primers for PCR were listed in Table 5.

2.8. Statistical analysis

All experiments were independently repeated in triplicate. Expression differences between different groups were analyzed using unpaired t-test and chi-square test. Data were expressed as mean ± SE. The data was processed by SPSS 22.0 software (SPSS, Chicago, IL, USA), and visualized by GraphPad Prism 6.0 (GraphPad Software Inc, CA, USA). P-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Identification of DEGs.

Eventually, a total of 2629 DEGs were screened out between NEC and control samples, consisting of 367 up-regulated genes and 2262 down-regulated genes. (Figure 1) All these DEGs above were enrolled in the present study.

3.2. Functional and pathways analysis of DEGs.

To reveal a more specific function pattern of these genes, functional enrichment analysis was conducted by DAVID. Since Biological Process (BP) in GO can better reflect the function of genes, we selected BP as the main term for the function enrichment analysis of DEGs. Among the 545 BP terms enriched involving DEGs, the top 10 GO terms are indicated in Table 1 according to the P value, including "oxidation reduction", "inflammatory response", etc. Moreover, the associated 39 pathways significantly enriched by DEGs were listed in Table 2 and these pathways were mainly related to metabolism, such as" Drug metabolism", "Tryptophan metabolism".

3.3.PPI network of DEGs.

By integrating DEG pairs with combined score > 0. 4, PPI network was constructed according to the STRING database and visualized in Cytoscape, involving 2323 nodes (DEGs) and 16777 edges (Figure 2), accounting for 88.36% of all DEGs. In this study, the whole network included a giant component and several disconnected nodes. The major central nodes controlling the flow in the network must lie in the giant network as the isolated nodes only occupied a small part which should be discarded. To analyze and progress the giant network conveniently, we extracted it from the whole network. As is shown in Figure 3, the topology property of the network indicates that the node degree distribution of the PPI was in power-law distribution. In this network, DEGs which have high connectivity degree, included P53(degree, 251), ACACB(degree, 159), IL6(degree, 157), DECR1(degree, 154), AGT(degree, 152), IL8(degree, 142), CDH1(degree, 124), CAT(degree, 121), KNG1(degree, 120), PPARA(degree, 128) etc. and thus they were defined as hub genes.

3.4. Module analysis of PPI network.

Biological networks are likely consisted of several functional modules in which the subunits of the complex and their interactions generally lead to the same biological process. This proposes a new perspective for the biological function of the various components comprising the complex network. Several modules were extracted from the PPI network through MCODE analysis.

The most highly connected cluster identified in our network consisted of 44 nodes and 946 interactions (cluster rank 1; Score 44) (Figure 4). Accordingly, AGT, IL8 and KNG1 were enriched in the above module that extracted from the PPI network of DEGs.

3.5.Disease risk sub‑pathway analysis.

A total of 189 disease risk sub‑pathways were identified as presented in Table 3, such as Tryptophan metabolism, Fatty acid metabolism, and Arachidonic acid metabolism. It should be noted that acetyl-Coenzyme A carboxylase beta(ACACB) and catalase(CAT) which were enriched in different sub‑pathways including propanoate metabolism and tryptophan metabolism also had a higher node degree according to the PPI network.

3.6. Expression of key genes in NEC

QRT-PCR was conducted to measure the expression of key genes in NEC samples. As shown in Figure 5A-E, the expression of IL-8 was up-regulated in NEC samples compared with healthy tissues, while the expression of ATG, KNG1, ACACB and CAT in NEC small intestine tissues were all lower than negative controls. The results revealed that these genes may be involved in the mechanism of NEC.

4. Discussion

NEC remains to be a major cause of acute morbidity and mortality for neonates as well as a cause of long term disability for older children. Although great efforts have been done on this disease, recent advances show no effective preventive strategy, and treatment options. Thus, there is an urgent need to explore the mechanism of NEC, and the knowledge gained would be a great help for further diagnoses and treatment strategies. In the present study, a total of 2629 DEGs, including 367 up-regulated genes and 2262 down-regulated genes were identified. Functional enrichment analysis was conducted to reveal the associated BP terms and pathways among the DEGs. Then, PPI network was constructed and several hub nodes were identified. According to the results of the PPI network analysis, the most highly connected module was identified and then AGT, IL8 and KNG1 which has high degrees were screened. Besides, ACACB and CAT were sorted to be involved in propanoate metabolism and tryptophan metabolism.

In the present study, AGT, was significantly enriched were down-regulated in module analysis of PPI. AGT, also named angiotensinogen, can be converted into angiotensin I by renin followed by generation of angiotensin II (AII) by the angiotensin‑converting enzyme (ACE).(Metwali et al., 1996). During the granulomatous inflammation

progress, the angiotensin plays an important but imprecise role which indicate that they may be involved in intestinal inflammation of Crohn's disease(Schulte et al., 2001). In addition, AII can also increase the generation of transforming growth factor β1 (TGFβ1), which can regulate the activity of inflammatory cytokines (Maheshwari et al., 2011). These results suggested that AGT may be associated with NEC progression in modulating of TGFβ1. In addition to AGT1, IL8 and KNG1 were also recognized as hub nodes in the present PPI network analysis. IL8 is a major member of the CXC chemokine family which can regulate the inflammatory response through recruiting neutrophils to the site of inflammation (Akdis et al., 2011) (Cho et al., 2016). IL8 mRNA was over-expressed in intestinal tissues of NEC infants compared with controls as well as serum IL-8, which can be regarded as a potential marker for NEC(Weitkamp et al., 2013).

Besides, KNG1 plays an important role in regulating blood pressure, inflammation and blood clotting through encoding high molecular weight kiniongen protein (HMWK). Bradykinin (BK) and high-molecular-weight kininogen (HKa) are cleavage products of HMWK. HKa promotes the progress of inflammatory diseases through releasing cytokines TNF-α, IL-1β, IL-6, and chemokines IL-8 and MCP-1 (Khan et al., 2006). HKa also plays an anti-adhesion effect, thereby recruiting leukocyte into inflamed tissue (Chavakis et al., 2001). All the above evidence suggests that KNG1 may exert a great influence in NEC.

In addition, these findings have shown that ACACB and CAT with high node degrees in PPI network are associated with propanoate and tryptophan metabolism pathways. Mitochondrial (ACC2) acetyl-CoA carboxylase, which is the encoding product of ACACB, acts as a key rate limiting enzyme of the regulation of mitochondrial fatty acid oxidation (Abu-Elheiga et al., 2001) (Wakil and Abu-Elheiga, 2009). It is currently considered to be one of the potential targets for regulating human disease in obesity, diabetes, cancer and cardiovascular complications (Ma et al., 2011). Previous study showed that continuous oxidation of fatty acid in the ACACB knock-out mice can lead to high insulin sensitivity (Oh et al., 2005). A change in tissue ACACB via transcriptional regulation may be of greater importance in the intestine involved in NEC. Through encoding catalase, CAT converts the reactive oxygen species hydrogen peroxide to water and oxygen, thereby reduces the toxic effects of hydrogen peroxide and eventually play a protective oxidative stress (Glorieux et al., 2015). Increasing studies of oxidative stress in NEC are in progress as oxidative stress is characterized by excessive amounts of oxidants or decreased levels of antioxidants (Perrone et al., 2014) (Ng et al., 2015). Hence, low expression of CAT may promote the occurrence of NEC through the weakening

of antioxidant stress.

Taken together, the present study has identified several key genes (AGT, IL8, KNG1, ACACB and CAT) were involved in the mechanism of NEC and qRT-PCR was conducted to identify the expression of these genes in NEC samples. AGT, IL8 and KNG1 might serve an essential role in the drug metabolism and tryptophan metabolism pathway in NEC. Besides, ACACB and CAT may be crucial for propanoate metabolism and tryptophan metabolism to NEC onset and progression. These results may provide a novel understanding of the mechanism of NEC. Further research of the genes identified in this study is urgently needed to determine their detail mechanism of NEC.

Acknowledgements

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Confilct of interest

The authors declare no conflict of interest.

References

Abu-Elheiga, L., Matzuk, M.M., Abo-Hashema, K.A. and Wakil, S.J., 2001. Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. Science 291, 2613-6.

Akdis, M., Burgler, S., Crameri, R., Eiwegger, T., Fujita, H., Gomez, E., Klunker, S., Meyer, N., O'Mahony, L., Palomares, O., Rhyner, C., Ouaked, N., Schaffartzik, A., Van De Veen, W., Zeller, S., Zimmermann, M. and Akdis, C.A., 2011. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. J Allergy Clin Immunol 127, 701-21 e1-70.

Bandettini, W.P., Kellman, P., Mancini, C., Booker, O.J., Vasu, S., Leung, S.W., Wilson, J.R., Shanbhag, S.M., Chen, M.Y. and Arai, A.E., 2012. MultiContrast Delayed Enhancement (MCODE) improves detection of subendocardial myocardial infarction by late gadolinium enhancement cardiovascular magnetic resonance: a clinical validation study. J Cardiovasc Magn Reson 14, 83.

Chan, K.Y., Leung, K.T., Tam, Y.H., Lam, H.S., Cheung, H.M., Ma, T.P., Lee, K.H., To, K.F., Li, K. and Ng, P.C., 2014. Genome-wide expression profiles of necrotizing enterocolitis versus spontaneous intestinal perforation in human intestinal tissues: dysregulation of functional pathways. Ann Surg 260, 1128-37.

Chavakis, T., Kanse, S.M., Pixley, R.A., May, A.E., Isordia-Salas, I., Colman, R.W. and Preissner, K.T., 2001. Regulation of leukocyte recruitment by polypeptides derived from high molecular weight kininogen. FASEB J 15, 2365-76.

Cho, S.X., Berger, P.J., Nold-Petry, C.A. and Nold, M.F., 2016. The immunological landscape in necrotising enterocolitis. Expert Rev Mol Med 18, e12.

Dennis, G., Jr., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C. and Lempicki, R.A., 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 4, P3.

Elgin, T.G., Kern, S.L. and McElroy, S.J., 2016. Development of the Neonatal Intestinal Microbiome and Its Association With Necrotizing Enterocolitis. Clin Ther 38, 706-15.

Glorieux, C., Zamocky, M., Sandoval, J.M., Verrax, J. and Calderon, P.B., 2015. Regulation of catalase expression in healthy and cancerous cells. Free Radic Biol Med 87, 84-97.

Henry, M.C. and Moss, R.L., 2009. Necrotizing enterocolitis. Annu Rev Med 60, 111-24.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U. and Speed, T.P., 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249-64.

Khan, M.M., Bradford, H.N., Isordia-Salas, I., Liu, Y., Wu, Y., Espinola, R.G., Ghebrehiwet, B. and Colman, R.W., 2006. High-molecular-weight kininogen fragments stimulate the secretion of cytokines and chemokines through uPAR, Mac-1, and gC1qR in monocytes. Arterioscler Thromb Vasc Biol 26, 2260-6.

Li, C., Li, X., Miao, Y., Wang, Q., Jiang, W., Xu, C., Li, J., Han, J., Zhang, F., Gong, B. and Xu, L., 2009. SubpathwayMiner: a software package for flexible identification of pathways. Nucleic Acids Res 37, e131.

Llanos, A.R., Moss, M.E., Pinzon, M.C., Dye, T., Sinkin, R.A. and Kendig, J.W., 2002. Epidemiology of neonatal necrotising enterocolitis: a population-based study. Paediatr Perinat Epidemiol 16, 342-9.

Ma, L., Mondal, A.K., Murea, M., Sharma, N.K., Tonjes, A., Langberg, K.A., Das, S.K., Franks, P.W., Kovacs, P., Antinozzi, P.A., Stumvoll, M., Parks, J.S., Elbein, S.C. and Freedman, B.I., 2011. The effect of ACACB cis-variants on gene expression and metabolic traits. PLoS One 6, e23860.

Maheshwari, A., Kelly, D.R., Nicola, T., Ambalavanan, N., Jain, S.K., Murphy-Ullrich, J., Athar, M., Shimamura, M., Bhandari, V., Aprahamian, C., Dimmitt, R.A., Serra, R. and Ohls, R.K., 2011. TGF-beta2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. Gastroenterology 140, 242-53.

Metwali, A., Elliott, D., Blum, A. and Weinstock, J.V., 1996. What Models of Granulomatous Inflammation Provide the Immunologist. Methods 9, 305-10.

Neu, J., 2014. Necrotizing enterocolitis: the mystery goes on. Neonatology 106, 289-95.

Ng, P.C., Chan, K.Y., Leung, K.T., Tam, Y.H., Ma, T.P., Lam, H.S., Cheung, H.M., Lee, K.H., To, K.F. and Li, K., 2015. Comparative MiRNA Expressional Profiles and Molecular Networks in Human Small Bowel Tissues of Necrotizing Enterocolitis and Spontaneous Intestinal Perforation. PLoS One 10, e0135737.

Oh, W., Abu-Elheiga, L., Kordari, P., Gu, Z., Shaikenov, T., Chirala, S.S. and Wakil, S.J., 2005. Glucose and fat metabolism in adipose tissue of acetyl-CoA carboxylase 2 knockout mice. Proc Natl Acad Sci U S A 102, 1384-9.

Perrone, S., Tataranno, M.L., Santacroce, A., Negro, S. and Buonocore, G., 2014. The role of oxidative stress on necrotizing enterocolitis in very low birth weight infants. Curr Pediatr Rev 10, 202-7.

Schulte, C.M., Goebell, H., Roher, H.D. and Schulte, K.M., 2001. C-509T polymorphism in the TGFB1 gene promoter: impact on Crohn's disease susceptibility and clinical course? Immunogenetics 53, 178-82.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B. and Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13, 2498-504.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3, Article3.

Somaschini, M., Breda-Klobus, A. and Pacati, I., 2012. [Necrotizing enterocolitis (nec): risk factors and genetic

susceptibility]. Minerva Pediatr 64, 33-40.

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K.P., Kuhn, M., Bork, P., Jensen, L.J. and von Mering, C., 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43, D447-52.

Thompson, A.M. and Bizzarro, M.J., 2008. Necrotizing enterocolitis in newborns: pathogenesis, prevention and management. Drugs 68, 1227-38.

Wakil, S.J. and Abu-Elheiga, L.A., 2009. Fatty acid metabolism: target for metabolic syndrome. J Lipid Res 50 Suppl, S138-43.

Weitkamp, J.H., Koyama, T., Rock, M.T., Correa, H., Goettel, J.A., Matta, P., Oswald-Richter, K., Rosen, M.J., Engelhardt, B.G., Moore, D.J. and Polk, D.B., 2013. Necrotising enterocolitis is characterised by disrupted immune regulation and diminished mucosal regulatory (FOXP3)/effector (CD4, CD8) T cell ratios. Gut 62, 73-82.

Figure Legends

Fig. 1. Heatmap shows the up- and down-regulated genes. The horizontal axis represents the sample name. Red represents the up-regulated gene while green represents the down-regulated gene.

Fig. 2. The giant network consisting of 2,323 nodes and 16,777 edges was extracted from the whole protein-protein interaction network. Key nodes in the giant network are highlighted in different colors: Red corresponds to the up-regulated gene and green corresponds to the down-regulated gene in NEC.

Fig. 3. The topology parameters were analyzed according to the giant networks. (A) Degree distribution; (B) Average clustering coefficient; (C) Shortest path distribution; (D) Closeness centrality.

Fig. 4. The most significant module was extracted from the giant network: Red corresponds to the up-regulated gene and green corresponds to the down-regulated gene in NEC.

Fig.5. The expression of key genes in NEC patients' tissues. AGT (A); IL-8 (B); KNG1 (C); ACACB (D); CAT (E).

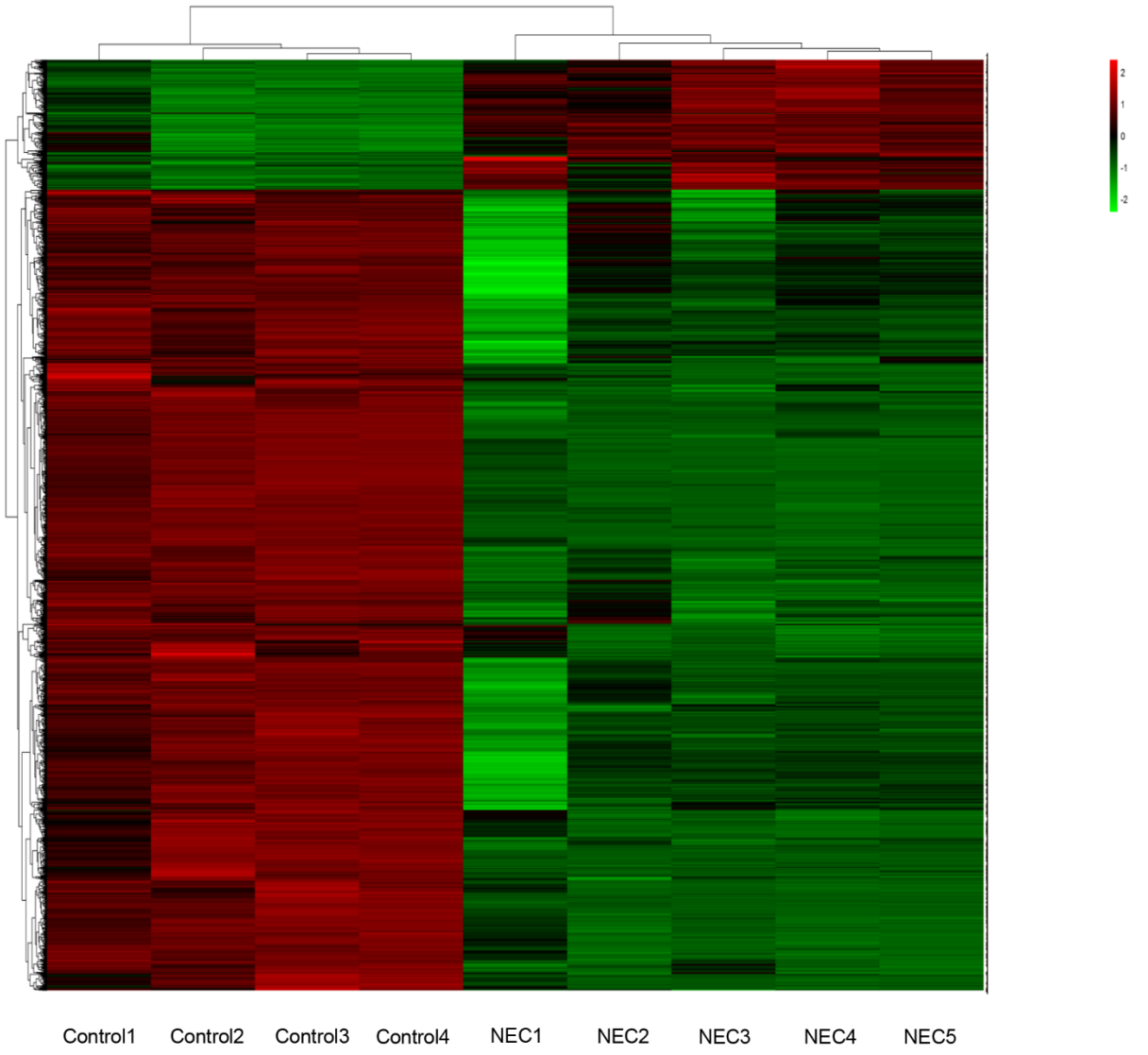

Fig.1

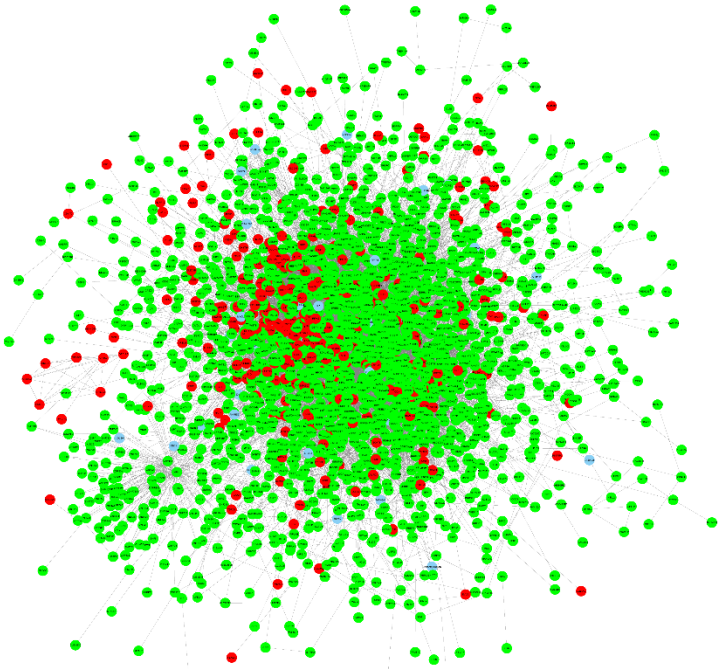

Fig.2

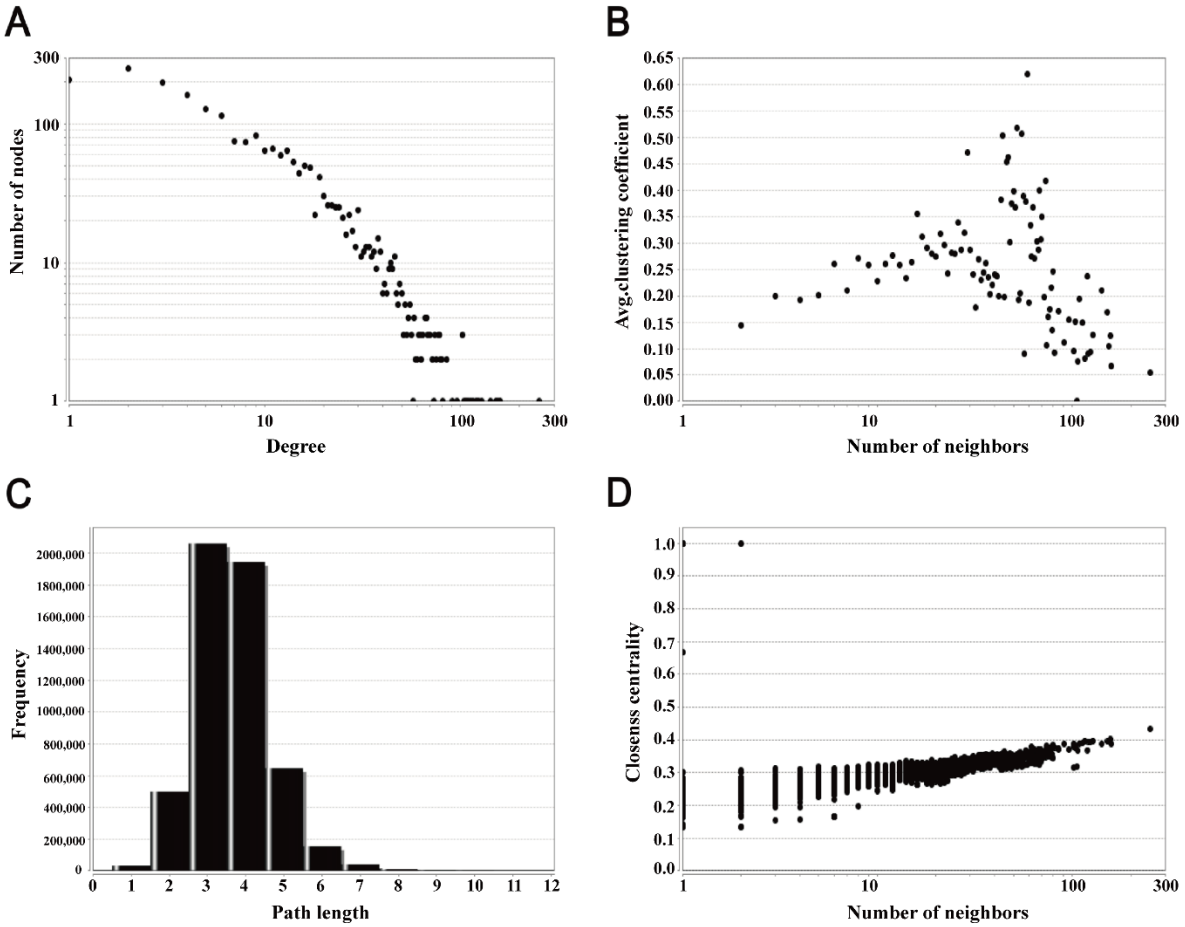

Fig.3

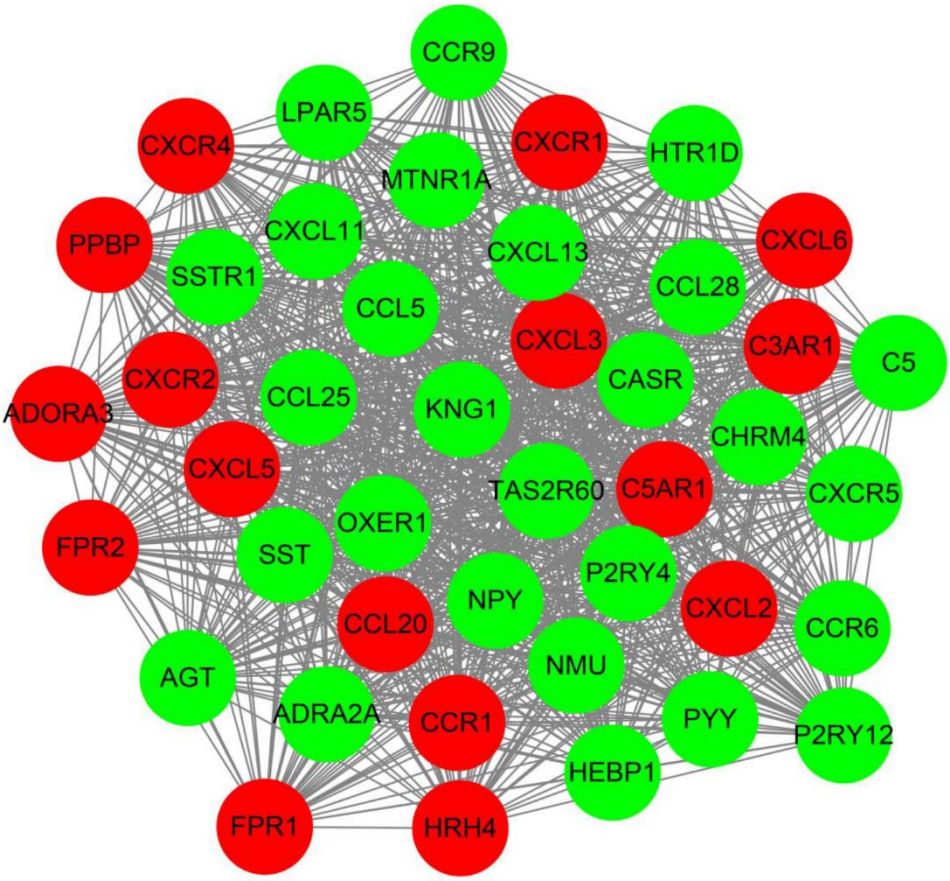

Fig.4

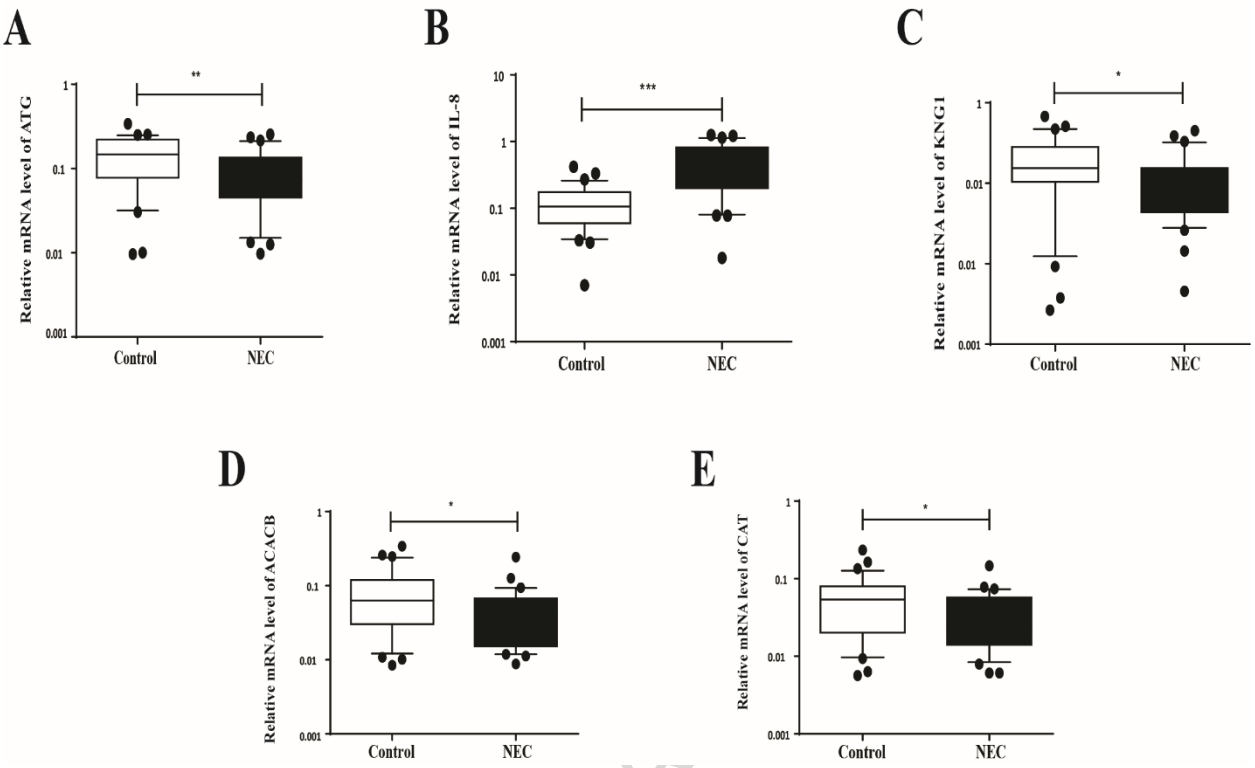

Fig.5

TableI. Partial results of enriched GO functional analyses of DEGs (Top 10)

Category	GO ID	GO Term	Count	PValue	FDR
BP	GO:0055114	oxidation reduction	175	3.31E-19	6.22E-16
BP	GO:0009611	response to wounding	144	2.16E-15	3.95E-12
BP	GO:0006954	inflammatory response	100	1.69E-14	3.17E-11
BP	GO:0006631	fatty acid metabolic process	69	4.84E-13	9.07E-10
BP	GO:0006952	defense response	152	1.58E-12	2.97E-09
BP	GO:0008202	steroid metabolic process	66	4.18E-11	7.84E-08
BP	GO:0042330	taxis	53	2.48E-09	4.66E-06
BP	GO:0006935	chemotaxis	53	2.48E-09	4.66E-06
BP	GO:0006732	coenzyme metabolic process	51	4.07E-09	7.63E-06
BP	GO:0042592	homeostatic process	163	9.19E-09	1.72E-05

TableII. Partial results of KEGG pathway enrichment analyses of DEGs (Top 10)

KEGG pathway	KEGG entry	Count	PValue	FDR
Drug metabolism	hsa00982	28	5.64E-07	7.02E-04
Tryptophan metabolism	hsa00380	20	5.76E-06	0.007171
Metabolism of xenobiotics by cytochrome P450	hsa00980	25	1.43E-05	0.0178
Fatty acid metabolism	hsa00071	19	2.58E-05	0.032099
Valine, leucine and isoleucine degradation	hsa00280	20	3.15E-05	0.039248
Arginine and proline metabolism	hsa00330	22	5.79E-05	0.072064
Propanoate metabolism	hsa00640	16	6.84E-05	0.085147
PPAR signaling pathway	hsa03320	26	6.88E-05	0.085633
Arachidonic acid metabolism	hsa00590	22	1.48E-04	0.184059
Butanoate metabolism	hsa00650	16	1.61E-04	0.200316

TableIII. The statistically significant sub-pathways identified of DEGs (Top 10).

Sub-pathwayId	Sub-pathwayName	Count	PValue	FDR
path:00380_1	Tryptophan metabolism	26	7.21E-11	3.48E-08
path:00380_4	Tryptophan metabolism	24	1.32E-10	3.48E-08
path:00590_6	Arachidonic acid metabolism	27	2.34E-10	3.48E-08
path:00071_2	Fatty acid metabolism	21	2.74E-10	3.48E-08
path:00380_12	Tryptophan metabolism	23	3.14E-10	3.48E-08
path:00590_1	Arachidonic acid metabolism	28	6.19E-10	5.73E-08
path:00380_5	Tryptophan metabolism	22	6.39E-09	5.06E-07
path:00980_1	Metabolism of xenobiotics by cytochrome P450	30	8.13E-09	5.64E-07
path:00380_3	Tryptophan metabolism	20	1.28E-08	7.89E-07
path:00590_3	Arachidonic acid metabolism	24	3.99E-08	2.07E-06

Table 4. **Clinical characteristics of study population**

Variable	Control(n=32)	NEC(n=32)	P value
Age(days,mean,SE)	21.16（2.03）	19.09（2.90）	0.56[a]
Weight(kg,mean,SE)	2.95（0.08）	2.74（0.11）	0.14[a]
Sex(%)			
Male	15（46.88）	18（56.25）	0.45[b]
Female	17（53.12）	14（43.75）	

a Student's t-test.

b Two-sided chi-squared test.

Table 5: Sequences of primers for qRT-PCR

AGT	F: 5'-ACAATGAGAGTACCTGTGAGCA-3'
	R: 5'-TCTTGGCCTGAATTGGAGCAG-3'
IL8	F: 5'-ACTGAGAGTGATTGAGAGTGGAC-3'
	R: 5'-AACCCTCTGCACCCAGTTTTC-3'
KNG1	F: 5'-TGCTCCAGGCTGCTACTAAGT-3'
	R: 5'-GGCTTCAGTTATGCGGTACAA-3'
ACACB	F: 5'-CAAGCCGATCACCAAGAGTAAA-3'
	R: 5'-CCCTGAGTTATCAGAGGCTGG-3'
CAT	F: 5'-TGGAGCTGGTAACCCAGTAGG-3'
	R: 5'-CCTTTGCCTTGGAGTATTTGGTA-3'
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3'
	R: 5'-GGATCTCGCTCCTGGAAGATG-3'

Abbreviations

NEC: Necrotizing enterocolitis

DEGs: Differentially expressed genes

GO: gene ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

DAVID: Database for Annotation, Visualization and Integrated Discovery

PPI: Protein-protein interaction

STRING: Search Tool for the Retrieval of Interacting Genes

Highlights

Differentially expressed genes were determined with data from GEO database between necrotizing enterocolitis(NEC) and control tissues.

Several key genes were identified from the PPI network and risk sub-pathway information.

Candidate key genes expression were confirmed in clinical samples for further validation.