Original Research

Application of Monte Carlo cross-validation to identify pathway cross-talk in neonatal sepsis

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Impact statement

To find the best pairs of pathways able to discriminate sepsis samples versus normal samples, an RF classifier, the DS obtained by DEGs of paired pathways significantly associated, and Monte Carlo cross-validation were applied in this paper. Ten pairs of pathways were probably able to discriminate neonates with sepsis versus normal uninfected neonates. Among them, the best two paired pathways ((7) IL-6 Signaling and Phospholipase C Signaling (PLC); (8) Glucocorticoid Receptor (GR) Signaling and Dendritic Cell Maturation) were identified according to analysis of extensive literature.

Abstract

To explore genetic pathway cross-talk in neonates with sepsis, an integrated approach was used in this paper. To explore the potential relationships between differently expressed genes between normal uninfected neonates and neonates with sepsis and pathways, genetic profiling and biologic signaling pathway were first integrated. For different pathways, the score was obtained based upon the genetic expression by quantitatively analyzing the pathway cross-talk. The paired pathways with high cross-talk were identified by random forest classification. The purpose of the work was to find the best pairs of pathways able to discriminate sepsis samples versus normal samples. The results found 10 pairs of pathways, which were probably able to discriminate neonates with sepsis versus normal uninfected neonates. Among them, the best two paired pathways were identified according to analysis of extensive literature.

Keywords: Neonatal sepsis, gene pathway cross-talk, integrated approach, random forest classification

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Introduction

Neonatal sepsis frequently occurs at 28 days after birth because of functional immaturity of the neonatal immune system, as well as reduced passage of maternal antibodies, which enhance the risk of infecting various bacterial, fungal as well as viral infections.[1,2] Neonatal sepsis can lead to high mortality and morbidity rates in newborns infants, particularly among preterm infants.[3,4] Although perinatal care have been improved constantly, the risk of neonatal sepsis remains high in less-developed countries, including China,[1] neonatal sepsis survivors may induce neurodevelopmental sequelae.[5] As detailed above, it is of necessity to explore more accurate and simple diagnosis and therapy for neonatal sepsis.

Molecular technology and Omics technology consist of genomics, proteomics, and metabolomics have been utilized for rapidly diagnose neonatal sepsis.[3,6] Microarray assay and genome-wide detection profiling have been conducted for the neonatal sepsis, which could further understand the sepsis on the genome-level, discover new biomarker, and novel targets and pathways.[7] Additionally, the genome-wide method could potentially decrease research bias. Interrogating the entire genome in an impartial way offered a chance to identify previously unrecognized, unconsidered targets, and signaling pathways associated with the biology of neonatal sepsis. Many unavoidable farraginous factors inherent make microarray studies of clinical neonatal sepsis difficult. Furthermore, gene signatures obtained by many gene-based classification methods were all based on the chosen genes, even if the functional products can mutually interact.[8] In addition, the chosen gene markers probably unravel superfluous information.[8] Thus, the overall classification performance may not be synergistically improved. Understanding the complex interaction among genes was the best pathway to overcome these limitations, which is one of the key challenges of the postgenomic era.[9,10] Thence, exploring the metabolic and signaling pathways associated with a certain

phenotype plays a vital role in correct understanding of high-throughput genomic assay. Several studies have shown that classifiers based on pathway achieve comparable or better classification performance than classifiers based on independent gene biomarkers.[11,12] As mentioned above, most currently available methods consider the pathways as independent mechanisms, and they do not focused on the relation between pathways, which is referred to as cross-talk. In a normal condition, many cellular signaling pathways were interconnected to maintain homeostasis.[13] However, the development of heterogeneous clinical syndromes, such as sepsis, might affect the pathway interaction and comprehensively alter the phenotype of a cell. Therefore, the interaction between different signaling pathways is a vital step for understanding the regulatory mechanisms and their synergistic effects on specific biologic procedures.

The innovative integrated method has been employed to explore the miRNAs functionally regulating genetic cross-talk pathway of aggressive breast carcinoma (BC).[8] To understand the gene signaling cross-talk pathway of neonatal sepsis, the new method is used in this study. Control and experimental group used in this study were selected as follows: control group was the uninfected neonates matched normal controls, and the experimental group was the newborn infants meeting criteria for sepsis based on specific definitions. Whole-blood-derived gene expression profiles of control group and experimental group were obtained. Furthermore, biological pathway was integrated to unravel the correlation between differentially expressed genes among different groups. For the paired signaling pathways, the score was calculated based upon the expression levels of multiples genes. Random forest classification method was applied to identify the pairs of high cross-talk pathways.

Experimental methods

Datasets

The sepsis datasets contain: (1) the expression levels of 15,449 genes (after quantile analysis) and 26 neonatal sepsis samples (experimental group) and 37 uninfected neonatal samples (control group) obtained with Illumina HumanHT-12 v4 expression BeadChip, which reports the normalized results for the expression of a gene.

The proposed methodology

The purpose of this research was to develop a method to quantify the cross-talk between pathways, which could differentiate the normal samples and sepsis samples. Figure 1 showed the proposed processes. All steps were repeated for 50 bootstraps in Monte Carlo Cross-Validation approach. In the approach, results including (1) DEG, (2) PEA, (3) Mean, (4) DScore, and (5) classification were obtained for each bootstrap. Top 10 pairs of signaling pathways with the optimal AUC (area under the concentration time curve) value and their corresponding genes were obtained and focused on after 50 bootstraps. The approach of this paper was

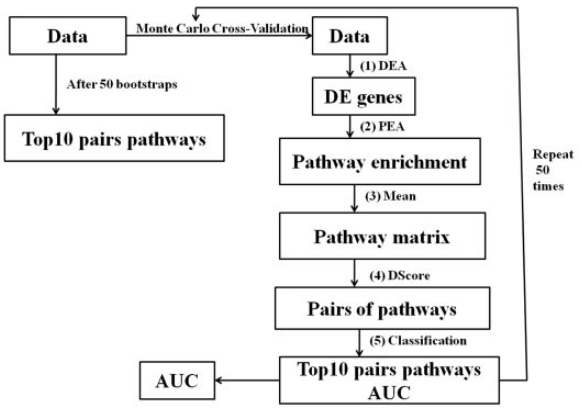

Figure 1. Workflow of the proposed methodology.

compared using the genomic biomarkers selected by the Monte Carlo Cross-Validation: key genes form DEG.

Step 1: Differential expression analysis.

DEG on those mRNA transcripts, which had mean across the 63 samples, higher than the 0.25* quantile mean across all samples, were applied. To confirm whether a certain gene expresses in a differential way, hypothesis testing and the fold-change among sepsis and normal conditions were applied.

Particularly, the edger package from Bioconductor, which used quantile-adjusted conditional maximum likelihood (qCML) approach during single factor test, was applied to determine genes with different expression levels.[14] qCML is the most reliable estimator compared to several other estimators, which is well acknowledged. The obtained *P* values were sorted in an ascending order, which are corrected using Benjamini-Hochberg producer for multiple testing correction.[15] If log fold change (FC) more than 1 or FDR less than 0.01, significant DEGs were considered to be existing between the experimental group and control group.

Step 2: Pathway enrichment analysis.

A pathway enrichment analysis from DEGs (PEA-DEGs) was used to explore different DEGs-enriched signaling pathways in experimental group with respect to control group. Furthermore, 977 biologic signaling pathways derived from the IPA (Ingenuity Pathways Analysis) tool (http://www.ingenuity.com/) were selected to be further investigated.

The enrichment degree was accessed by Fisher's exact test. The aim was to place DEGs under a regulating circumstance (IPA pathways) and identify the pathways responsible for regulating the activity, thereby emphasizing the regulating apparatus which promotes phenotypic differentiation. A Fisher's test was applied between DEGs and genes related to IPA signaling pathways. We thus obtained pathway enriched with P value <0.01 values. The Benjamini-Hochberg process for multi-testing correction was applied to adjust the P values.[15] IPA pathways were filtered based on the genes obtained from the quantile function.

Steps 3–4: Discriminating score for pathway cross-talk.

Discriminating score (DS) was computed by analyzing the expression levels of paired DEGs-enriched IPA pathways (e.g. we applied a DS (x, y) in every sample for the paired pathways: x, y). DS was defined as DS =(Mx − My)/(Sx+ Sy), (1) where Mx and Sx represent average value and standard deviation in a pathway x and My and Sy in a pathway y. DS score defines the correlation between paired pathways. A larger value represents higher level of difference of activity among signaling pathways.[16] The considered DSs were already used in previous studies for comparing expression between different subgroups presenting amplification and the subgroup of sample with no amplification. Herein, the DS was used to analyze pathway cross-talk. DS was compared with the method proposed by Cava *et al.*,[17] which uses the Euclidean distance as metric to quantify pathway cross-talk.

Step 5: Identification of the best paired pathways.

To evaluate the performance of the proposed methodology, a Random Forest classification model was developed using the R-package.[18] The model was used to classify the considered experimental group versus control group. AUC value was assessed by cross-validation method ($k = 10$). Classification was applied on pairs of pathways using DS for each sample.

Monte Carlo cross-validation approach was implemented in the further analysis. It chose some fraction of expression profile data (60% of original dataset) by random, to establish a training set, allocated the rest points to testing set, which were repeated multiple times (50 bootstraps). The DEGs, pathways PEA-DEGs, and a matrix score for paired signaling pathways were analyzed for each bootstrap. The analysis process is summarized in Figure 1. Pairs of pathway significantly enriched from DEGs and from a matrix score (DS) were obtained for each bootstrap; RF classifier establishes an AUC value. Thus, the top 10 pairs of pathway for each bootstrap that obtained the best classification performance in the training dataset were considered. A testing dataset was then used to validate the top 10 pairs of pathways for each bootstrap. After all 50 bootstraps (runs), we selected a list of the top 10 paired pathway sequencing according to a decreasing frequency that each pair of pathway was selected in the 50 runs.

Each pathway with respect to their AUC was descending ordered and the first 10 pathways with the optimal 10 AUC were got. Moreover, top 10 pairs with better ranked AUC value for all 50 bootstraps were further analyzed. Figure 1 shows how the selection of the top 10 paired pathways is performed for each bootstrap and it also shows the final result after 50 bootstraps in the training dataset.

Results

A total of 15,449 genes were obtained via quantile analysis; 2076 DEGs were found between sepsis samples and normal samples via DEA, and a list of 88 significantly pathways enriched from DEGs in total represented a list of 154 unique genes. Pathways significantly enriched by DEGs with their FDR score ($FDR < 0.05$) were showed in Supplemental Table. The gene quantity in every signaling pathway, and number of shared genes between genes and DEGs in the pathway were also displayed (Supplemental Table).

The results for each bootstrap are shown in Figure 2. This is a Monte Carlo analysis. One row represents a pair of pathways. If there is a relationship, it is yellow, blue is irrelevant, and each row represents an experiment in a Monte Carlo analysis. The Monte Carlo scheme is an alpha dog Algorithm, so it can self-game, a column is the result of a self-game. We obtained a heatmap, where yellow squares reveal paired pathways at top 10 positions of the training dataset for the bootstrap. We found (1) Airway pathology in chronic obstructive pulmonary disease and role of NFAT in regulation of the immune response in eight bootstraps; (2) Inhibition of angiogenesis by TSP1 and airway pathology in chronic obstructive pulmonary disease in eight bootstraps; (3) Melatonin degradation III and airway pathology in chronic obstructive pulmonary disease in seven bootstraps; (4) Airway pathology in chronic obstructive pulmonary disease and dendritic cell maturation in six bootstraps; (5) Bladder cancer signaling and airway pathology in chronic obstructive pulmonary disease in six bootstraps; (6) Airway pathology in chronic obstructive pulmonary disease; differential regulation of cytokine production in intestinal epithelial cells by IL-17A and IL-17F in 6 bootstraps.

Each paired pathway was descending ordered according to their AUC and 10 paired pathways with $AUC > 0.90$ between sepsis samples and normal samples were found and focused on Table 1. These top 10 paired signaling pathways have the best performance for classification of sepsis samples and NS for all 50 bootstraps, which were listed as followed: (1) the pair of pathways Rapoport–Luebering Glycolytic Shunt and Phospholipase C Signaling; (2) the pair of pathways Role of NFAT in Regulation of the Immune Response and Hepatic Fibrosis/Hepatic Stellate Cell Activation; (3) Agranulocyte Adhesion and Diapedesis and Phospholipase C Signaling; (4) Superpathway of Melatonin Degradation and Atherosclerosis Signaling; (5) Glucocorticoid Receptor Signaling and Hepatic Fibrosis/Hepatic Stellate Cell Activation; (6) Glucocorticoid Receptor Signaling and Agranulocyte Adhesion and Diapedesis; (7) IL-6 Signaling and Phospholipase C Signaling; (8) Glucocorticoid Receptor Signaling and Dendritic Cell Maturation; (9) Superpathway of Melatonin Degradation and LXR/RXR Activation; and (10) Glucocorticoid Receptor Signaling and iCOS-iCOSL Signaling in T Helper Cells. Figure 3 shows overall AUC performances when we give only top 10 paired signaling pathways for each bootstrap after 50 bootstraps.

Discussion

In this paper, an RF classifier, the DS obtained by DEGs-paired pathways intimately associated, and Monte Carlo cross-validation were applied to identify the top 10 paired signaling pathways that obtained the optimal classification of all the considered bootstraps (50). The top 10 paired signaling pathways of an $AUC > 0.90$ among experimental group and control group were identified and

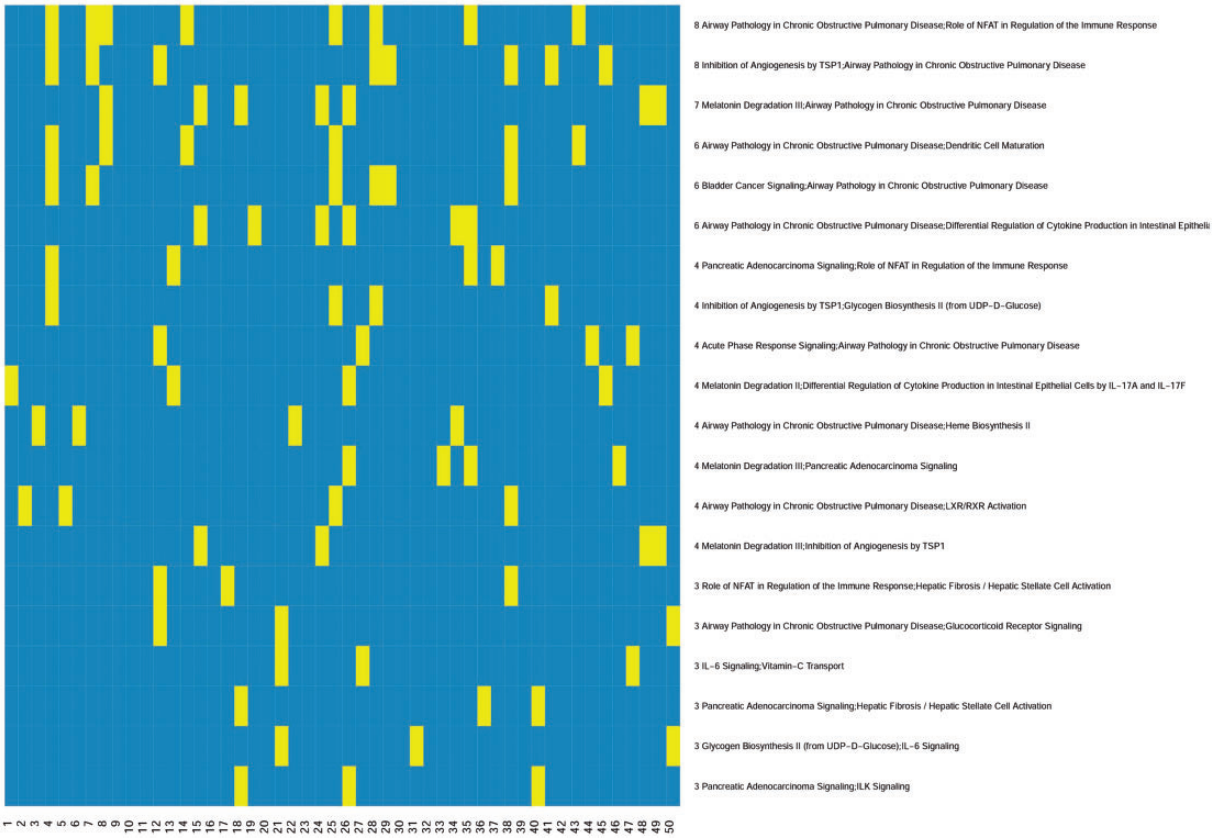

Figure 2. Heatmap with top 10 pairs of pathways (yellow square) that obtained the best AUC values in the training dataset.

Table 1. Top 10 pairs of pathways with AUC value for TCGA testing and GEO dataset.

Pairs of pathways	AUC value
Rapoport–Luebering Glycolytic Shunt; Phospholipase C Signaling	0.961
Role of NFAT in Regulation of the Immune Response; Hepatic Fibrosis/Hepatic Stellate Cell Activation	0.925
Agranulocyte Adhesion and Diapedesis; Phospholipase C Signaling	0.92
Superpathway of Melatonin Degradation; Atherosclerosis Signaling	0.918
Glucocorticoid Receptor Signaling; Hepatic Fibrosis/Hepatic Stellate Cell Activation	0.912
Glucocorticoid Receptor Signaling; Agranulocyte Adhesion and Diapedesis	0.908
IL-6 Signaling; Phospholipase C Signaling	0.908
Glucocorticoid receptor signaling; dendritic cell maturation	0.906
Superpathway of melatonin degradation; LXR/RXR activation	0.902
Glucocorticoid receptor signaling; iCOS-iCOSL signaling in T helper cells	0.902

AUC: area under the ROC curve; TCGA: the cancer genome atlas; GEO: Gene Expression Omnibus.

concerned. The top 10 paired signaling pathways contain 12 pathways affecting neonatal sepsis are detailed and analyzed hereinafter.

Parpoport–Luebering glycolytic shunt and phospholipase C signaling

Whether Parpoport–Luebering glycolytic shunt affects sepsis has not been reported currently. Activation of phospholipase C (PLC) inhibits the expression levels of IL-1β and TNF-α in lipopolysaccharide- (LPS) induced sepsis.[19] PLC activates the phospholipase A2 (PLA2), PLD, and MAPKs via stimulating the activation of protein kinase C (PKC) and the generation of intracellular calcium.[19,20] No report shows that PLC affect neonatal sepsis, but activity and secretion of PLA2 are increased in neonatal sepsis.[21] Therefore, whether Parpoport–Luebering glycolytic shunt and PLC signaling participate in neonatal sepsis remain unclear.

Effect of NFAT on immune actions and activation of hepatic stellate cell and hepatic fibrosis

Thus, calcium/nuclear factor of activated T cells (NFAT) signaling participates in the abdominal sepsis, which might be a useful target to treat abdominal sepsis.[22] However, no report shows that NFAT signaling participates in neonatal sepsis. Hepatic fibrosis is related to chronic inflammation in an acute-chronic sepsis mouse model.[23] Relatedly, Wnt5a, secreted by hepatic stellate cells (HSCs),

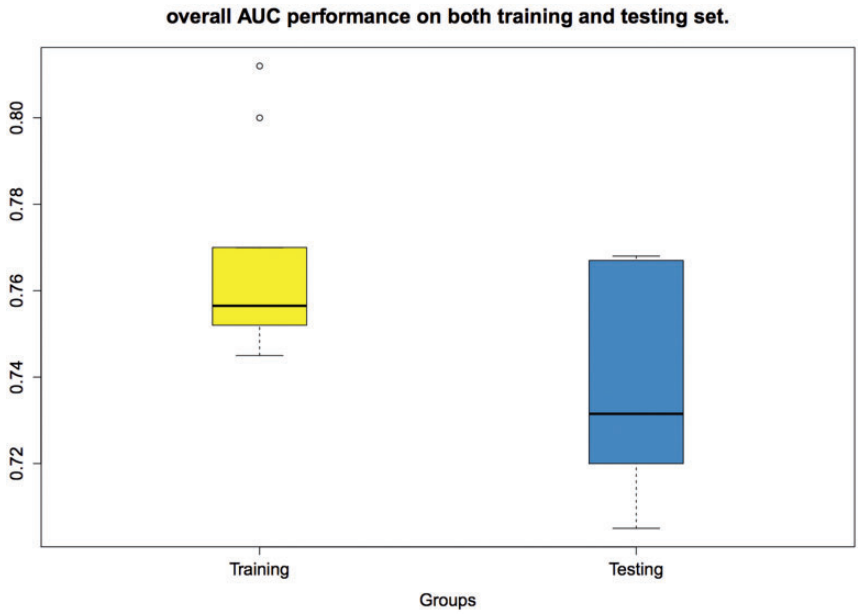

Figure 3. Boxplot: overall AUC (area under the ROC curve) performance on both training (yellow) and testing dataset (blue).

could activate the NF-κB and/or NFAT-dependent signaling pathway.[24] Therefore, NFAT-dependent signaling pathway may be associated with hepatic fibrosis/hepatic stellate cell activation via Wnt5a. Only some paper reported that neonatal sepsis could induce liver disease, no reports show that hepatic fibrosis/hepatic stellate cell activation acts on the neonatal sepsis.

Agranulocyte adhesion and diapedesis: Phospholipase C signaling

Phospholipase C (PLC) is involved in agranulocyte adhesion and diapedesis which is one of the important immune pathways.[25] However, no reports indicate that agranulocyte adhesion and diapedesis and PLC signaling indeed participate in neonatal sepsis, which needs more evidences.

Superpathway of melatonin degradation and atherosclerosis signaling

The expression of endogenous melatonin is up-regulated in patients with late onset sepsis (LOS).[26] Melatonin can treat neonatal sepsis, improving the clinical and laboratory outcomes.[26] Inactivated LXR/RXR complex can cause atherosclerosis, which is related to the incidence of severe sepsis.[27] Previous studies have prompted that melatonin may exert effects on multiple cardiovascular diseases, including atherosclerosis.[28] No report shows that atherosclerosis signaling influences neonatal sepsis. Thence, the paired pathways Superpathway of Melatonin Degradation and Atherosclerosis Signaling probably act together on the neonatal sepsis.

Glucocorticoid receptor signaling and hepatic stellate cell and hepatic fibrosis

Glucocorticoids (GCs) have been adopted in treating sepsis for more than five decades.[29] However, GCs significantly undermined the effect of antibiotic treatment on chorioamnionitis and neonatal sepsis.[29] Glucocorticoid receptors (GRs) isoform (GR-β) does not bind glucocorticoids.[29] Previous study showed that the expression level of GR-β in white blood cells collected from nine individuals diagnosed with septic shock was considerably up-regulated when admitted, which suggested that septic shock restrained the GCs activity by increasing expression status of negative GR-β. GCs and their receptors regulate the hepatic fibrosis via interacting with the TGF-β signaling pathway, which further suggests that hepatic fibrosis/hepatic stellate cell is associated with sepsis.[30] However, whether GR signal and hepatic fibrosis/hepatic stellate cell acts together on neonatal sepsis needs further investigation.

GR signaling and agranulocyte adhesion and diapedesis

A variety of studies have been shown that GR signaling participates in the sepsis, even neonatal sepsis. However, no reports suggest that agranulocyte adhesion and diapedesis are associated with GR signaling and neonatal sepsis currently. Therefore, we speculate that the paired pathway GR signaling and agranulocyte adhesion and diapedesis are probably not important for neonatal sepsis.

IL-6 signaling and phospholipase C signaling

Plasma concentrations of inflammatory cytokines are increased in neonatal sepsis, which can be utilized to early monitor serious septic shock or bacterial sepsis.[6] No report shows that PLC affects neonatal sepsis, but activity and secretion of PLA2 are increased in neonatal sepsis.[31] PLC activates the PLA2, PLD, and MAPKs via stimulating PKC and enhancing intracellular calcium. PLC probably regulates the secretion of IL-6 via activating PLD and

PKC in neonatal sepsis,[32,33] which needs further investigation.

GR signaling and dendritic cell maturation

Loss and decreases of dendritic cells (DCs) maturation have been noted in patients and models with sepsis.[34] The estimation of DCs number and maturation state may be used as prognostic markers of neonatal sepsis. Endogenous GCs mitigate LPS-inducing inflammatory response and accelerate the tolerance by preventing the expression levels of IL-12 and DC in mouse models without the GR in DCs (GRCD11c-cre).[31] Thus, GR signaling and DC maturation are associated with each other and acts together on neonatal sepsis.

Superpathway of melatonin degradation and LXR/RXR activation

Activated retinoid X receptor/X receptor/(RXR/LXR) in liver can down-regulate expression levels of pro-inflammatory cytokines via suppressing the NF-κB activity. LXR/RXR activation can be inhibited by severe sepsis.[35] Inactivated LXR/RXR complex can induce atherosclerosis, which instead causes severe sepsis.[36] Both LXR/RXR activation and superpathway of melatonin degradation are associated with atherosclerosis signaling and sepsis. However, the relationship between LXR/RXR activation and superpathway of melatonin degradation remains unclear. Whether LXR/RXR activation has an effect on neonatal sepsis also needs further investigations.

GR signaling and iCOS-iCOSL signaling in T helper cells

T helper cell, such as Th1, Th2, and Th17 as well as their secreted cytokines participate in the innate immunity and neonatal sepsis.[37] Inducible costimulator (iCOS) and iCOS ligand (iCOSL) participate in the activation of T cells. No reports showed that iCOS-iCOSL signaling had effect on neonatal sepsis. GR signaling plays important roles in neonatal sepsis. There is no relation between GR signaling and iCOS-iCOSL signaling in T helper cells. Therefore, the paired pathway of GR signaling and iCOS-iCOSL signaling in T helper cells probably does not act together on neonatal sepsis.

Only two paired pathways, which probably affected neonatal sepsis and were able to discriminate neonates with sepsis versus uninfected neonates, were identified according to analysis of extensive literature. The two paired pathways were listed as follows: (7) IL-6 Signaling and Phospholipase C Signaling (PLC); (8) GR Signaling and Dendritic Cell Maturation. In addition, three paired pathways ((2) the pair of pathways Role of NFAT in Regulation of the Immune Response and Hepatic Fibrosis/Hepatic Stellate Cell Activation; (4) Superpathway of Melatonin Degradation and Atherosclerosis Signaling; (5) GR Signaling and Hepatic Fibrosis/Hepatic Stellate Cell Activation probably play important roles in sepsis. More evidences were needed to investigate whether the three paired pathways affect neonatal sepsis.

Authors' contributions: YZ and JW performed literature review and conceived the study. XL and CL were in charge of protocol design, ethical approval, patient enrollment and statistical analysis. YZ completed the manuscript draft. All authors have reviewed and approved the final manuscript.

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