


RESEARCH ARTICLE

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Long noncoding RNA and messenger RNA abnormalities in pediatric sepsis: a preliminary study

Zhenjiang Bai^{1†}, Yiping Li^{2†}, Yanhong Li^{2,3†}, Jian Pan², Jian Wang² and Fang Fang^{2*} 

Abstract

Background: Sepsis represents a complex disease with dysregulated inflammatory response and high mortality rate. Long noncoding RNAs (lncRNAs) have been reported to play regulatory roles in a variety of biological processes. However, studies evaluating the function of lncRNAs in pediatric sepsis are scarce, and current knowledge of the role of lncRNAs in pediatric sepsis is still limited. The present study explored the expression patterns of both lncRNAs and mRNAs between pediatric sepsis patients and healthy controls based on a comprehensive microarray analysis.

Methods: lncRNA and mRNA microarray was used to detect the expression of lncRNAs and mRNAs in the septic and control groups. Aberrantly expressed mRNAs and lncRNAs identified were further interpreted by enrichment analysis, receiver operating characteristic (ROC) curve analysis, co-expression network analysis, and quantitative real-time PCR (qPCR).

Results: A total of 1488 differentially expressed lncRNAs and 1460 differentially expressed mRNAs were identified. A co-expression network of the identified lncRNAs and mRNAs was constructed. In this network, lncRNA lnc-RP11-1220 K2.2.1-7 is correlated with mRNA CXCR1 and CLEC4D; lncRNA lnc-ANXA3-2 is correlated with mRNA CLEC4D; lncRNA lnc-TRAPPC5-1 is correlated with mRNA DYSF and HLX; lncRNA lnc-ZNF638-1 is correlated with mRNA DYSF and HLX. Significantly different expressions between pediatric sepsis patients and controls were validated by qPCR for the 4 lncRNAs and 4 co-expressed mRNAs, validating the microarray results.

Conclusions: Our study contributes to a comprehensive understanding of the involvement of lncRNAs and mRNAs in pediatric sepsis, which may guide subsequent experimental research. Furthermore, our study may also provide potential candidate lncRNAs and mRNAs for the diagnosis and treatment of pediatric sepsis.

Keywords: Pediatric sepsis, Long non-coding RNA, Messenger RNA, Expression profile

Background

Long noncoding RNAs (lncRNAs) have been reported to play regulatory roles in a variety of biological processes [1–4]. Recent studies have shown that abnormal expressions of lncRNAs are involved in many inflammation-

related diseases [5–9]. Sepsis represents a complex disease with dysregulated inflammatory response and high mortality rate. It is the world's leading killer of children [10]. However, studies evaluating the function of lncRNAs in pediatric sepsis are scarce, and current knowledge of the role of lncRNAs in pediatric sepsis is still limited.

In the current study, we investigated the expression patterns of both lncRNAs and mRNAs between pediatric

* Correspondence: baseff77@hotmail.com

[†]Zhenjiang Bai, Yiping Li and Yanhong Li contributed equally to this work.

²Institute of Pediatric Research, Children's Hospital of Soochow University, Suzhou, China

Full list of author information is available at the end of the article



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sepsis patients and healthy controls based on a comprehensive microarray analysis. Aberrantly expressed mRNAs and lncRNAs identified were further interpreted by enrichment analysis, receiver operating characteristic (ROC) curve analysis, co-expression network analysis, and quantitative real-time PCR (qPCR). Our research contributes to a comprehensive understanding of the involvement of lncRNAs and mRNAs in pediatric sepsis, which may guide subsequent experimental research. Furthermore, our study may also provide potential candidate lncRNAs and mRNAs for the diagnosis and treatment of pediatric sepsis.

Methods

Sample preparation

Ten pediatric sepsis patients and 12 children who were scheduled for minor elective surgery such as circumcision or inguinal hernia repair as the control group were included (Table 1, Additional file 1). All the heparinized blood samples were obtained from Children's Hospital of Soochow University. The study procedure was approved by the ethics committee of Children's Hospital of Soochow University. The written informed consent was obtained from each participating individual's guardian. Mononuclear cells (MNCs) were isolated, and then stored at -80°C before RNA extraction. The comparison of clinical characteristics between study groups was carried out using Mann–Whitney U test for continuous variables and Fisher's exact test for categorical variables.

lncRNA and mRNA microarray

Total RNA was extracted using RNAiso (TaKaRa, Dalian, China) from the 10 septic children and 12 controls. Affymetrix Human oelncRNA Array (v1.0, containing

91,363 lncRNAs and 27,134 coding transcripts) was used to detect the expression of lncRNAs and mRNAs in the septic and control groups. Total RNAs purity and concentration were evaluated by NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA integrity was detected by capillary electrophoresis. According to manufacturers' instructions, sample preparation, microarray labeling and hybridization were performed. In brief, total RNAs from cells were reverse transcribed to double strand cDNAs and then synthesized, labeled, and hybridized onto the lncRNA and mRNA microarray.

Data analysis methods

Raw data generated using the Affymetrix Human oelncRNA Array from 12 normal controls and 10 septic patients were stored in CEL files, and then pre-processed (background correction, quantile normalization, log₂ transformed) using the Robust Multichip Average (RMA) method of the R package "affy" [11]. The R package "limma" was used to identify differentially expressed transcripts [12, 13] according to the criteria: (a) absolute Log₂ Fold Change (LFC) was more than 1; (b) for the Wilcoxon test false discovery rate (FDR)-adjusted *P*-value was less than 0.05.

Functional interpretation (both gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis) of the differentially expressed genes identified was further carried out using DAVID 6.8 [14, 15]. In GO analysis, in order to identify significantly enriched GO terms, a *P* value threshold of 0.05 was used [16]. In pathway analysis, enrichment analysis was performed by the hypergeometric test with a *P* value threshold of 0.05 based on the KEGG database [17]. ROC curves were used to assess the classification performance of lncRNAs and single coding genes [18]. In

Table 1 Clinical characteristics of the sepsis and control groups used in both microarray and qPCR validation

Characteristic	Sepsis	Control	<i>P</i>
Number	10	12	
Age, median months [range]	4.32 [1.17–96.60]	12.95 [5.80–20.13]	0.10 ^a
Gender			0.57 ^b
Male	8	11	
Female	2	1	
Infection site			
Lung (%)	2 (20.0)	–	–
Brain (%)	4 (40.0)	–	–
Others (%)	4 (40.0)	–	–
Septic shock (%)	3 (30.0)	–	–
ICU stay, median hours [range]	273.50 [23.00, 1009.00]	–	–
ICU mortality (%)	3 (30.0)	–	–

^a *P* value of the Mann–Whitney U test

^b *P* value of the Fisher's exact test

addition, according to the expression levels, differentially expressed lncRNAs and mRNAs were also calculated using Pearson correlation coefficient. The correlation threshold was set to > 0.98. Then, the eligible correlated lncRNA-mRNA pairs were chosen to construct a co-expression network by Cytoscape 3.4.0 software [19]. Moreover, conservation analysis was also performed using the R package “phastCons100way.UCSC.hg19” [20–22].

Quantitative real-time PCR validation

The remaining portion of microarray samples was used for quantitative real-time PCR validation. The RNA was reverse-transcribed using oligo-dT and mouse mammary tumor virus reverse transcriptase. qPCR was carried out with SYBR Green master mix. Primers designed were listed in Additional file 2. Transcript expression was normalized to β-actin mRNA. Relative expression of transcript was calculated according to the 2^{-ΔΔCt} method. Mann–Whitney U test was carried out to determine the expression difference between septic children and control group. Statistical analyses were carried out using GraphPad Prism software (GraphPad Software Inc.). All P values are two-sided. P < 0.05 was considered as statistically significant.

Results

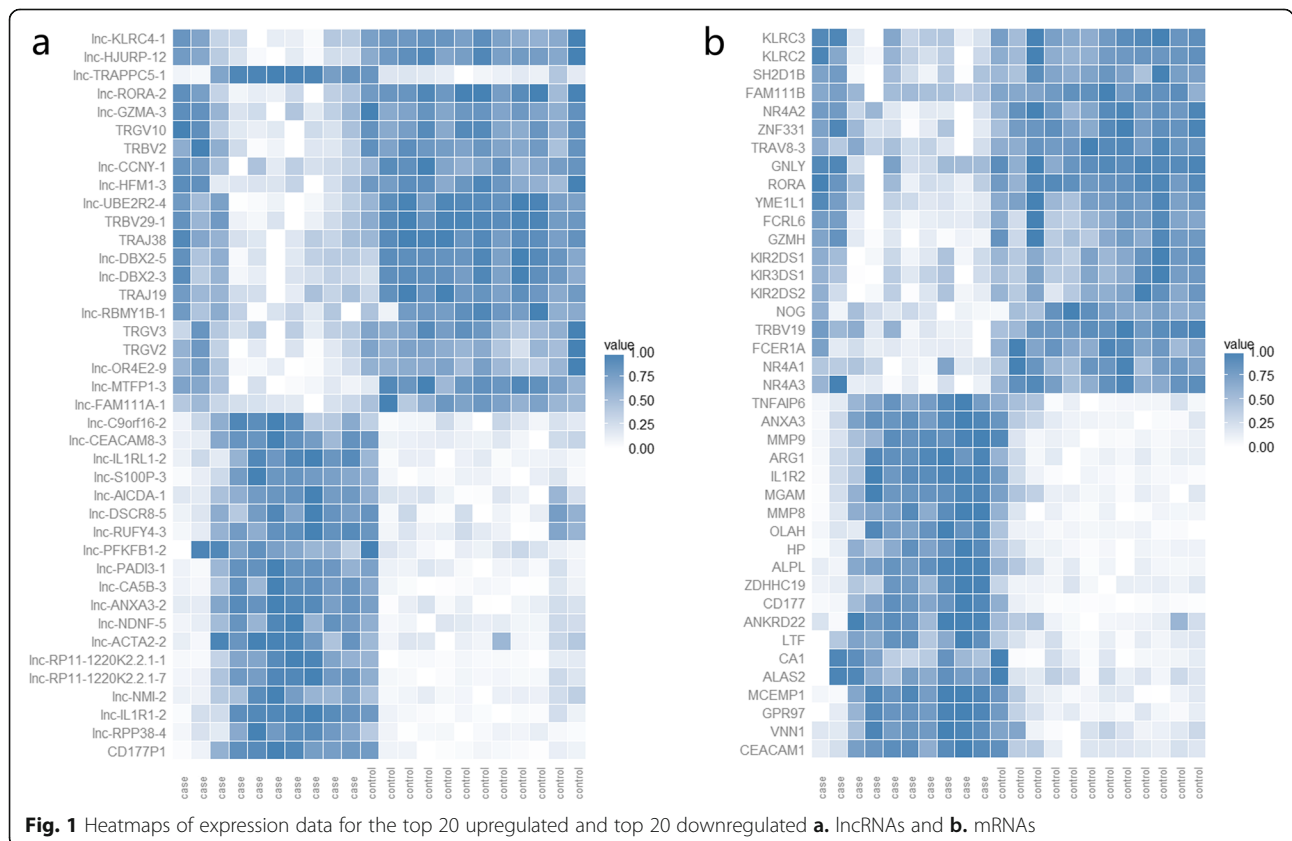
Differentially expressed lncRNAs and mRNAs identified

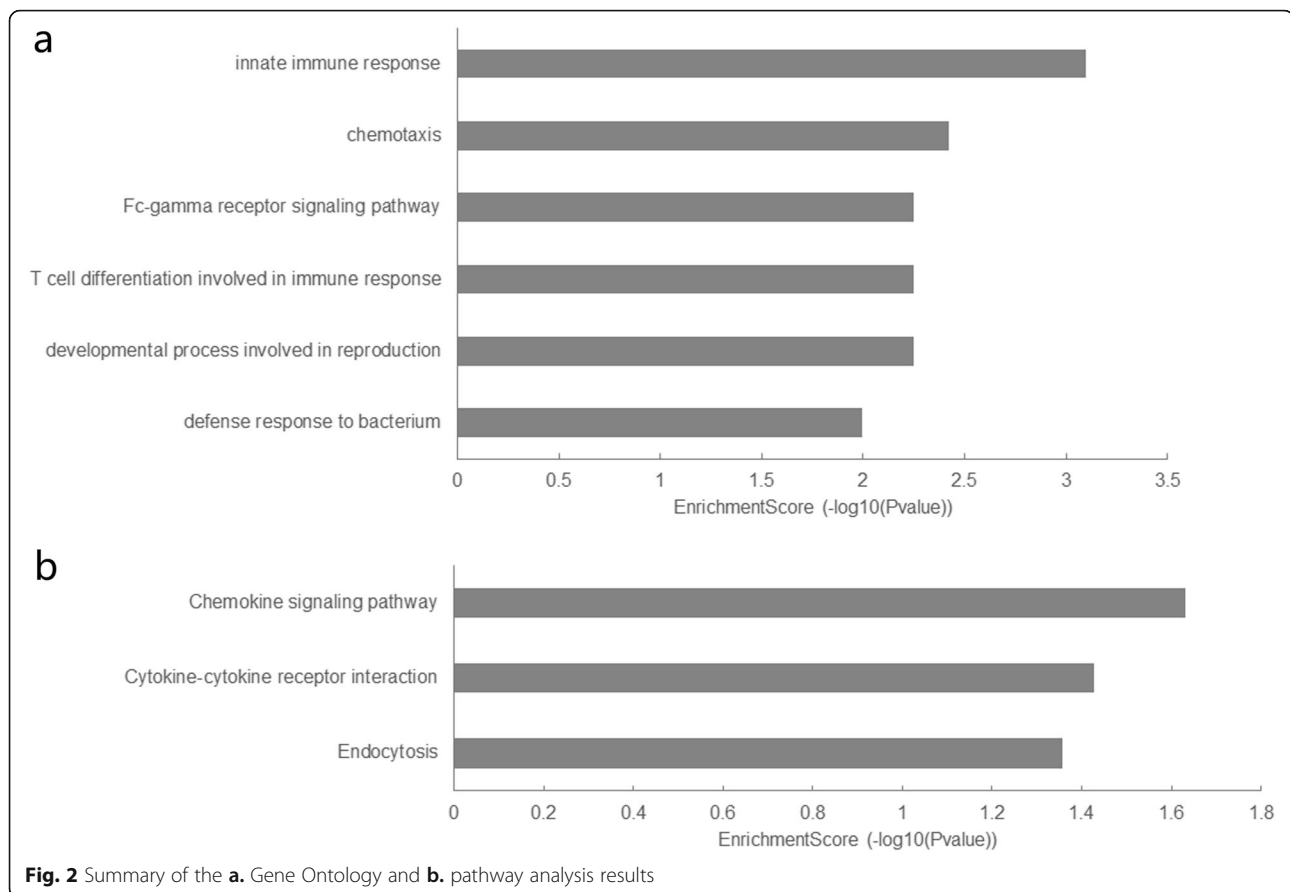
Ninety-one thousand three hundred sixty-three lncRNAs in total were detected, among which 1488 differentially expressed lncRNAs (996 upregulated; 492 downregulated, Additional file 3) were identified from 10 pediatric sepsis patients and 12 normal controls using the Affymetrix Human oelncRNA Array. The top 20 upregulated and top 20 downregulated lncRNAs are shown in Fig. 1a. lnc-RPP38–4 and lnc-HFM1–3 were the most upregulated and downregulated lncRNAs.

A total of 1460 differentially expressed mRNAs were also identified (1018 upregulated; 442 downregulated, Additional file 4) using the Affymetrix Human oelncRNA Array between pediatric sepsis group and control group. The top 20 upregulated and downregulated mRNAs are shown in Fig. 1b. MMP8 and FCER1A were the most upregulated and downregulated mRNAs.

Functional annotation of differentially expressed lncRNAs and mRNAs

The 1460 differentially expressed mRNAs underwent further functional investigation (GO analysis and pathway analysis). Figure 2 presented a summary of the GO and pathway analysis results. In the GO analysis, the top GO biological process term enriched was “innate immune





response”. In the pathway analysis, the most significant pathway identified was chemokine signaling pathway, when we mapped the mRNAs to the KEGG database.

As for diagnostic prediction quality, ROC analysis results indicated that the top performing mRNAs were NLRC3, TMEM204, SPPL2A, UBASH3A, ARHGAP29, FKBP9, MTUS1, LCK, DAAM2 (Additional file 5), and the top performing lncRNAs were lnc-FAM111A-1, TRBV29-1, lnc-UBE2R2-4, lnc-RPP38-4, lnc-IL1RL1-2, lnc-RORA-2, TRAJ19, lnc-HJURP-12, lnc-RP11-1220 K2.2.1-7 (Fig. 3).

lncRNA-mRNA co-expression network

A co-expression network of lncRNAs and mRNAs was constructed using highly correlated transcript pairs (correlation coefficients > 0.98), which included 47 nodes and 177 edges (Fig. 4). As shown in Fig. 4, lncRNA lnc-RP11-1220 K2.2.1-7 is correlated with mRNA C-X-C motif chemokine receptor 1 (CXCR1) and C-type lectin domain family 4 member D (CLEC4D); lncRNA lnc-ANXA3-2 is correlated with mRNA CLEC4D; lncRNA lnc-TRAPPC5-1 is correlated with mRNA dysferlin (DYSF) and H2.0 like homeobox (HLX); lncRNA lnc-ZNF638-1 is correlated with mRNA DYSF and HLX. Conservation analysis was

carried out and the conservation scores of the 4 lncRNAs (lncRNA lnc-RP11-1220 K2.2.1-7, lncRNA lnc-ANXA3-2, lncRNA lnc-TRAPPC5-1, lncRNA lnc-ZNF638-1) indicate weak conservations (Additional file 6).

Enrichment analysis of the co-expression network was also performed. The GO analysis results suggested that mRNAs in the co-expression network were enriched in innate immune response, chemotaxis, defense response to bacterium, interleukin-8-mediated signaling pathway, inflammatory response, T cell differentiation involved in immune response, Fc-gamma receptor signaling pathway, developmental process involved in reproduction, leukocyte migration, and dendritic cell chemotaxis. These results implicated that lncRNAs of the co-expression network correlated with mRNA expression and involved in the pathogenesis of sepsis.

lncRNA & mRNA expression was validated by qPCR

To validate the expression changes of lncRNAs and mRNAs in pediatric sepsis detected by microarray, the 4 lncRNAs (lncRNA lnc-RP11-1220 K2.2.1-7, lncRNA lnc-ANXA3-2, lncRNA lnc-TRAPPC5-1, lncRNA lnc-ZNF638-1) and 4 co-expressed mRNAs (CXCR1, CLEC4D, DYSF, HLX) in the co-expression network (see

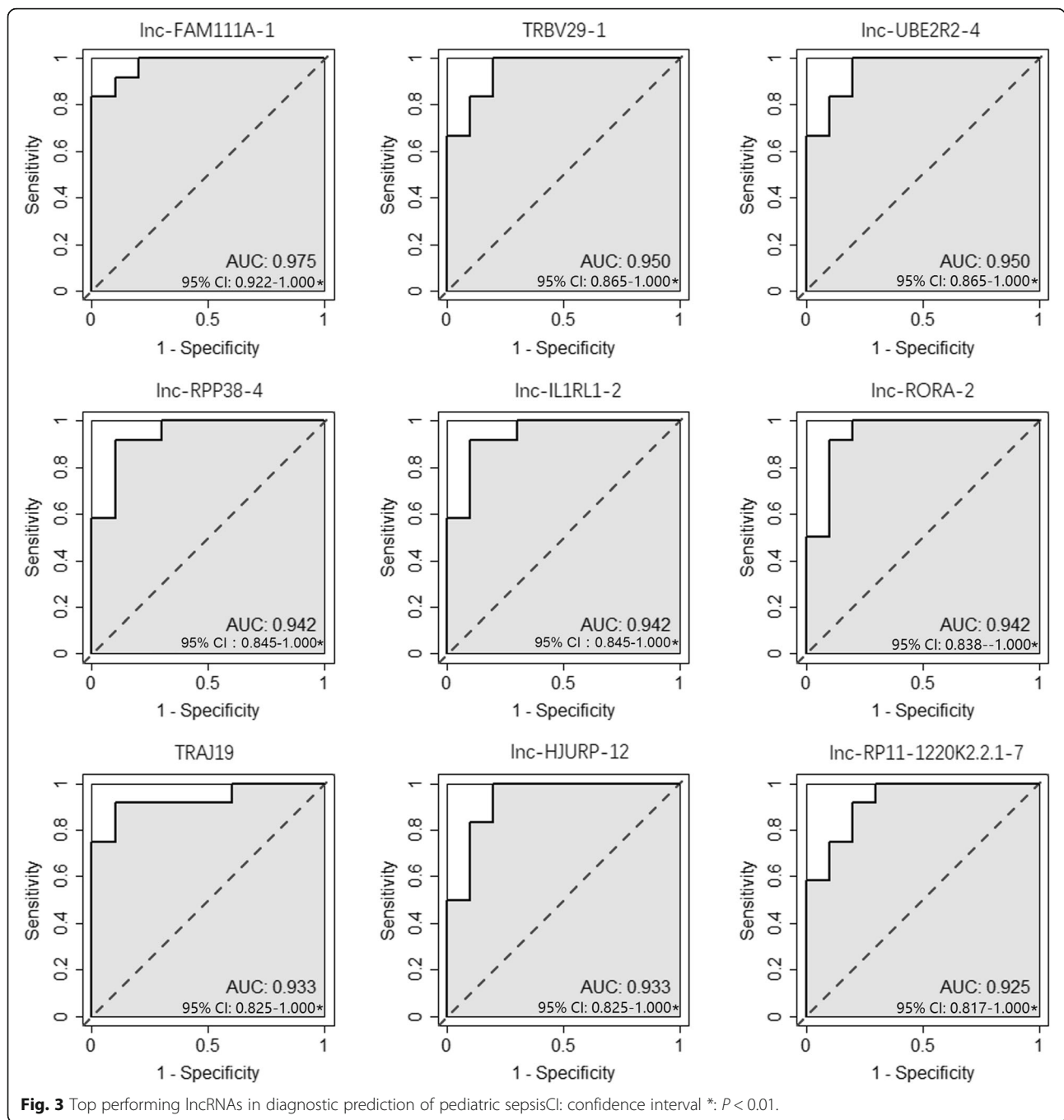
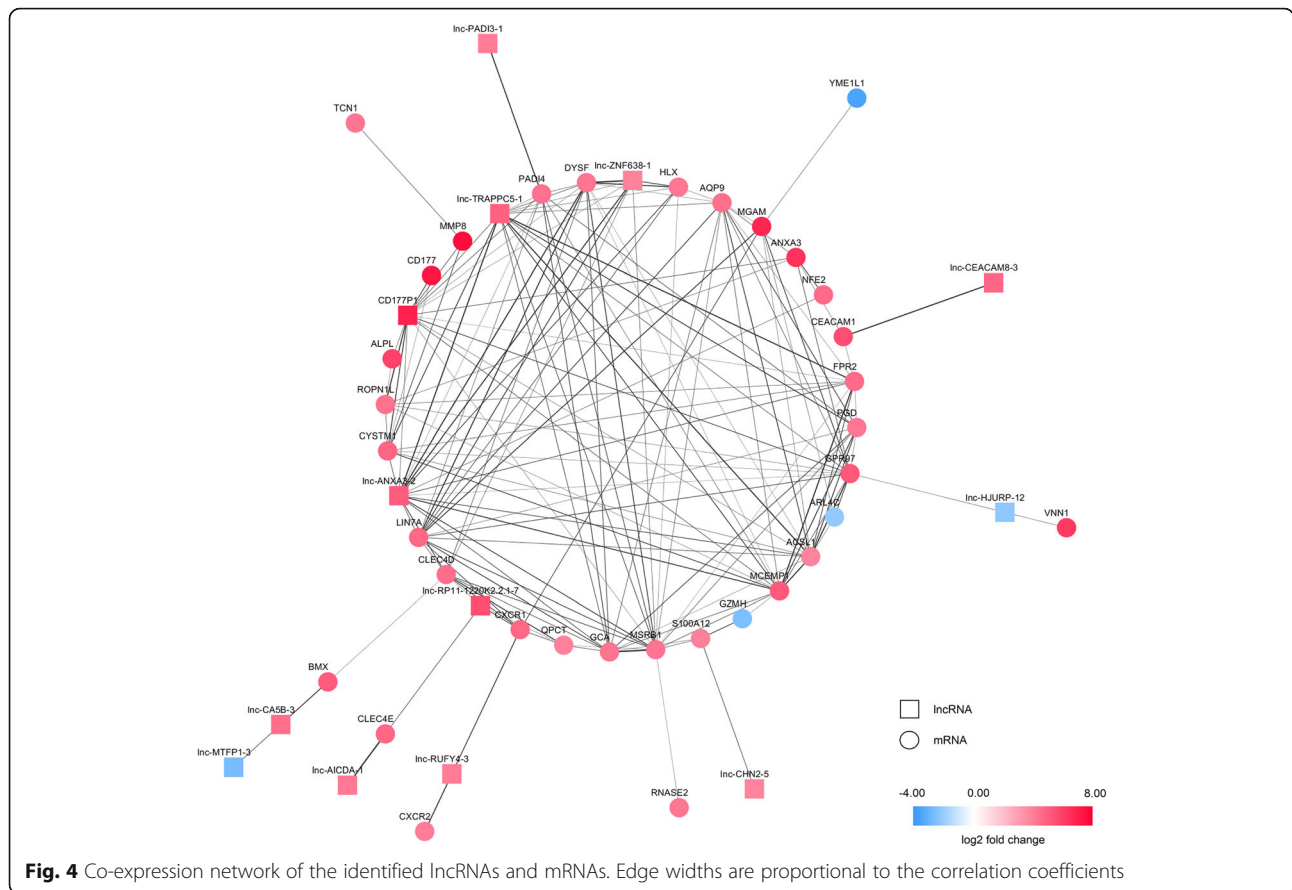


Fig. 4) were further selected for qPCR validation using the remaining portion of microarray samples. As presented in Fig. 5, the expression levels of the 4 lncRNAs and 4 mRNAs in the sepsis group were significantly higher than those of the control group ($P < 0.01$ respectively, see Fig. 5a and Fig. 5b). The qPCR results were similar to those acquired from microarray (Fig. 5c), suggest that the 4 lncRNAs (lncRNA Inc-RP11-1220 K2.2.1-7, lncRNA Inc-ANXA3-2, lncRNA Inc-TRAPPC5-1, lncRNA Inc-

ZNF638-1) are significantly highly expressed in septic children and could be novel biomarkers for pediatric sepsis.

Discussion

Recent researches suggest that abnormal expressions of lncRNAs play roles in many inflammation-related diseases [5-9]. Comprehensive microarray analysis enables both lncRNA and mRNA screening in pediatric sepsis,

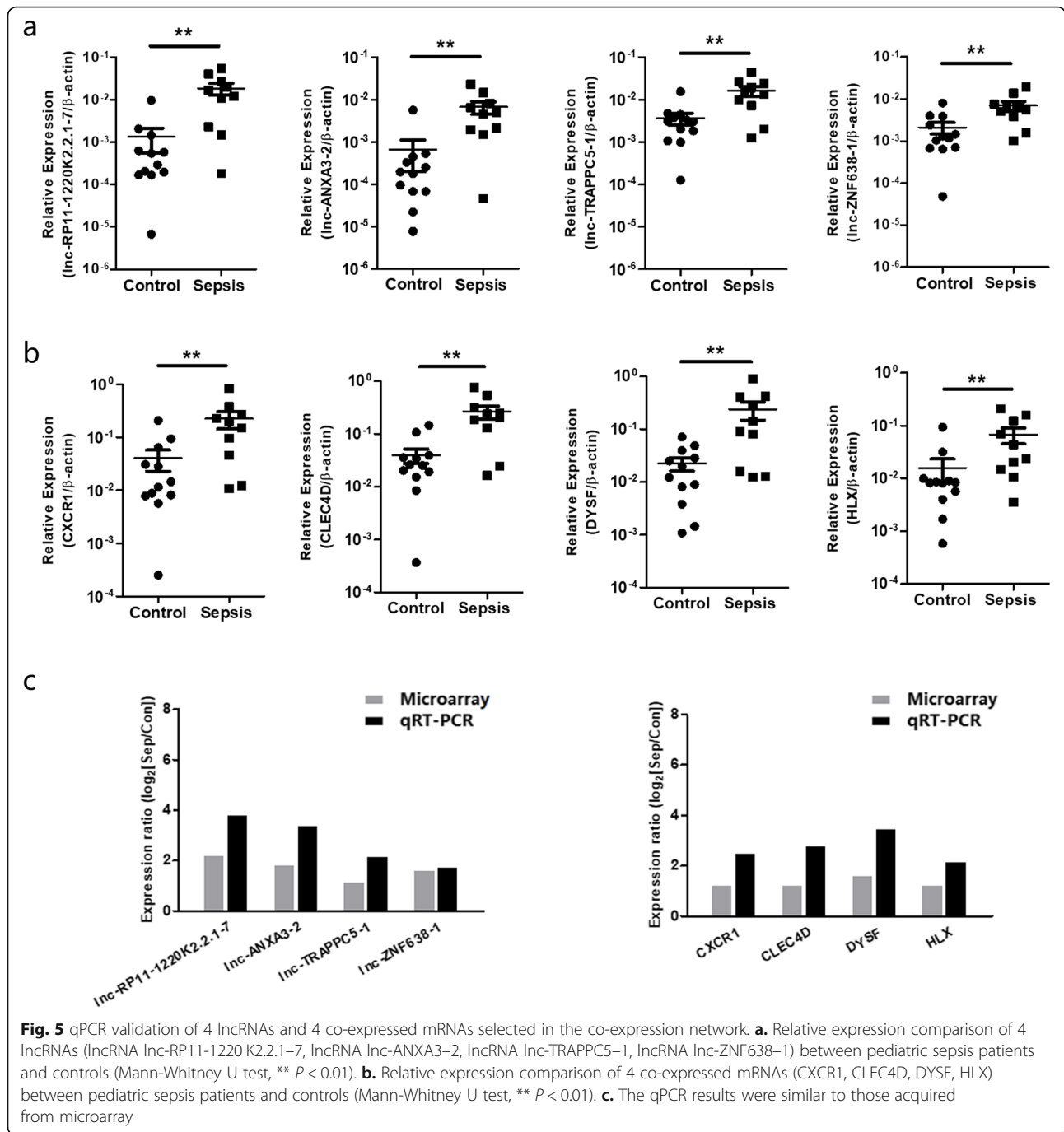


which has the likelihood of discovering candidate lncRNAs and mRNAs for diagnosis and treatment.

In this study, 1488 differentially expressed lncRNAs and 1460 differentially expressed mRNAs were identified using the Affymetrix Human oelncRNA Array. A co-expression network of the identified lncRNAs and mRNAs was constructed. In this network, lncRNA lnc-RP11-1220 K2.2.1-7 is correlated with mRNA CXCR1 and CLEC4D; lncRNA lnc-ANXA3-2 is correlated with mRNA CLEC4D; lncRNA lnc-TRAPPC5-1 is correlated with mRNA DYSF and HLX; lncRNA lnc-ZNF638-1 is correlated with mRNA DYSF and HLX. Significantly different expressions between pediatric sepsis patients and controls were validated by qPCR for the 4 lncRNAs (lncRNA lnc-RP11-1220 K2.2.1-7, lncRNA lnc-ANXA3-2, lncRNA lnc-TRAPPC5-1, lncRNA lnc-ZNF638-1) and 4 co-expressed mRNAs (CXCR1, CLEC4D, DYSF, HLX), validating the microarray results. CXCR1 is one of the major chemokine receptors on polymorphonuclear neutrophils [23], and the involvement of polymorphonuclear neutrophils in sepsis is well recognized [24–27]. CLEC4D, a member of Dectin-2 family, functions in resolution of inflammation, possibly through facilitating neutrophil turnover [28]. DYSF, a type II transmembrane protein, is involved in

muscle membrane repair [29, 30], and also functions in the regulation of cellular adhesion in human monocytes [31]. HLX is known to be a marker of immature hematopoietic cells, and it also plays a role in the activation of T lymphocyte or natural killer cells [32, 33]. HLX is reported to be involved in the macrophage differentiation process as well [34]. Enrichment analysis of the co-expression network suggested that mRNAs in the co-expression network were enriched in innate immune response. These results indicated that lncRNAs of the co-expression network correlated with mRNA expression and involved in the pathogenesis of sepsis. Compared with prior study - FEBS Open Bio. 2019; 9(1): 148–158, our analysis identified novel lncRNA – mRNA pairs that play roles in sepsis.

In addition, it is important to note that this study has several limitations. First, the small sample size required more careful consideration on our analysis. Second, considering the influence of gender and age on pediatric sepsis patients has been reported [35], subgroup analyses based on potential influential factors (such as age, gender, and disease severity) are needed in future research. Third, little is known about the molecular functions of these candidate lncRNAs



in the development of pediatric sepsis, more in vivo / in vitro research is therefore needed to be carried out in the future.

Conclusions

Here, we reported a total of 1488 differentially expressed lncRNAs and 1460 differentially expressed mRNAs in pediatric patients with sepsis. A co-expression network of these lncRNAs and mRNAs

was constructed and suggested that lncRNAs of the co-expression network correlated with mRNA expression and involved in the pathogenesis of sepsis. Our study contributes to a comprehensive understanding of the involvement of lncRNAs and mRNAs in pediatric sepsis, which may guide subsequent experimental research. Furthermore, our study may also provide potential candidate lncRNAs and mRNAs for the diagnosis and treatment of pediatric sepsis.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12920-020-0698-x>.

Additional file 1. Clinical parameters of the sepsis and control groups used in both microarray and qPCR validation.

Additional file 2. Primers designed for validation of lncRNA & mRNA expression patterns by qPCR.

Additional file 3. 1488 differentially expressed lncRNAs (996 upregulated; 492 downregulated) identified using the Affymetrix Human oelncRNA Array between pediatric sepsis group and control group.

Additional file 4. 1460 differentially expressed mRNAs (1018 upregulated; 442 downregulated) identified using the Affymetrix Human oelncRNA Array between pediatric sepsis group and control group.

Additional file 5. Top performing mRNAs in diagnostic prediction of pediatric sepsis.

Additional file 6. Conservation analysis results of the 4 lncRNAs.

Abbreviations

AUC: Area under the curve; CLEC4D: C-type lectin domain family 4 member D; CXCR1: C-X-C motif chemokine receptor 1; DYSF: Dysferlin; FDR: False discovery rate; GO: Gene ontology; HLX: H2.0 like homeobox; KEGG: Kyoto Encyclopedia of Genes and Genomes; LFC: Log₂ Fold Change; lncRNAs: Long noncoding RNAs; MNCs: Mononuclear cells; qPCR: Quantitative real-time PCR; RMA: Robust Multichip Average; ROC: Receiver operating characteristic

Acknowledgements

Not applicable.

Authors' contributions

FF designed the study, analyzed the data and drafted the manuscript; ZJB, YPL and YHL acquired patient data and performed laboratory experiments; JP and JW contributed to the study design and writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available in Gene Expression Omnibus (GEO) database [GSE145227].

Ethics approval and consent to participate

The study procedure was approved by the ethics committee of Children's Hospital of Soochow University, and written informed consent was obtained from each participating individual's guardian.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Pediatric Intensive Care Unit, Children's Hospital of Soochow University, Suzhou, China. ²Institute of Pediatric Research, Children's Hospital of

Soochow University, Suzhou, China. ³Department of Nephrology, Children's Hospital of Soochow University, Suzhou, China.

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